

POLYPEPTIDES COMPRISING MODIFIED IL-2 POLYPEPTIDES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority of US Provisional Application No. 62/789,075, filed January 7, 2019, which is incorporated by reference herein in its entirety for any purpose.

FIELD

[0002] The present invention relates to modified IL-2 with reduced affinity to CD25 and CD122 and such modified IL-2 fused to targeting moieties. The invention also relates to methods of using modified IL-2 and polypeptides comprising modified IL-2, including, but not limited to, methods of treating cancer.

BACKGROUND

[0003] IL-2 is a potent cytokine that stimulates T and NK cell proliferation through either a heterotrimeric IL-2 receptor (IL-2R) composed of CD25, CD122 and CD132, or a heterodimeric IL-2 receptor composed of only CD122 and CD132. Both forms of the IL-2R are potent mediators of T cell survival, proliferation, and overall activation status. IL-2 is generally produced by T cells and NK cells upon activation and mediates signaling in cis and trans in the local microenvironment. IL-2R signaling can induce differentiation of naïve T cells into effector and memory T cells, and can also stimulate suppressive regulatory T cells. Although the trimeric form of the IL-2R has a higher affinity for IL-2 than the dimeric form, both are reasonably high affinity and cause rapid receptor mediated internalization and degradation, resulting in an extremely short half-life. Recombinant human IL-2 (rhIL-2, Proleukin) is used clinically to treat renal cell carcinoma and malignant melanoma; however, it is associated with severe toxicity. Vascular leak syndrome is a major toxicity concern for cancer patients treated with Proleukin due to the effects of IL-2 signaling on endothelial cells that express the high affinity IL-2R.

[0004] T cells are activated through ligation of their TCR with a neighboring cell presenting MHC with complementary peptide bound, causing clustering of the TCR complex and signaling through NFAT. Co-stimulation of T cells through CD28 is driven by CD80 and CD86, which enhances T cell activation. After the initial activation, T cells upregulate a variety of proteins, including cytokine receptors as well as many co-stimulatory and checkpoint receptors that serve to modulate the T cell response.

[0005] Durable anti-tumor clinical responses have recently been reported for antagonist antibodies to checkpoint receptors, such as CTLA-4, PD-1, and PD-L1. However, even in the

most responsive indications the response rate is limited to about 30% of patients. Accordingly, there is a need for improved T cell modulating therapeutics.

SUMMARY

[0006] Provided herein are polypeptides comprising a modified IL-2 comprising at least one substitution at at least one amino acid position. In some examples, the modified IL-2 has reduced binding affinity for CD25, CD122, and/or an IL-2R relative to wild type IL-2. In some examples, the modified IL-2 has reduced activity on resting or activated T cells relative to wild type IL-2.

[0006a] In one aspect, the present disclosure refers to a polypeptide comprising a modified IL-2, wherein the modified IL-2 comprises a P65R substitution, a H16A substitution, and a substitution at amino acid position D84, wherein the amino acid positions correspond to the amino acid positions in SEQ ID NO: 1.

[0006b] In one aspect, the present disclosure refers to a complex comprising a first polypeptide and a second polypeptide, wherein the first polypeptide is the polypeptide disclosed herein.

[0006c] In one aspect, the present disclosure refers to a pharmaceutical composition comprising a polypeptide disclosed herein or the complex disclosed herein and a pharmaceutically acceptable carrier, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

[0006d] In one aspect, the present disclosure refers to an isolated nucleic acid that encodes a polypeptide disclosed herein or the complex disclosed herein.

[0006e] In one aspect, the present disclosure refers to an expression vector comprising the nucleic acid disclosed herein.

[0006f] In one aspect, the present disclosure refers to an isolated host cell comprising the nucleic acid disclosed herein or the expression vector disclosed herein.

[0006g] In one aspect, the present disclosure refers to an isolated host cell that expresses the polypeptide disclosed herein or the complex disclosed herein.

[0006h] In one aspect, the present disclosure refers to a method of producing the polypeptide disclosed herein or the complex disclosed herein comprising incubating the host cell disclosed herein under conditions suitable to express the polypeptide or complex, and optionally isolating the polypeptide or complex.

[0006i] In one aspect, the present disclosure refers to a method of increasing CD4+ and/or CD8+ T cell proliferation comprising contacting T cells with the polypeptide disclosed herein or the complex disclosed herein, wherein the CD4+ and/or CD8+ T cells are *in vitro*, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

[0006j] In one aspect, the present disclosure refers to the use of the polypeptide disclosed herein or the complex disclosed herein in the manufacture of a medicament for increasing CD4+ and/or CD8+ T cell proliferation *in vivo*, wherein the T cells are to be contacted with the medicament, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

[0006k] In one aspect, the present disclosure refers to an *in vitro* method of increasing NK cell proliferation comprising contacting NK cells with the polypeptide disclosed herein or the complex disclosed herein, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

[0006l] In one aspect, the present disclosure refers to the use of the polypeptide disclosed herein or the complex disclosed herein in the manufacture of a medicament for increasing NK cell proliferation *in vivo*, wherein the NK cells are to be contacted with the medicament, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

[0006m] In one aspect, the present disclosure refers to the use of the polypeptide disclosed herein or the complex disclosed herein, or the pharmaceutical composition disclosed herein in the manufacture of a medicament for treating a subject with cancer, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

Example 1. A polypeptide comprising a modified IL-2, wherein the modified IL-2 comprises at least one substitution at at least one amino acid position selected from P65, D84, E95, M23, and H16.

Example 2. The polypeptide of example 1, wherein the modified IL-2 is a modified human IL-2.

Example 3. The polypeptide of example 1 or example 2, wherein the amino acid positions correspond to the amino acid positions in SEQ ID NO: 1.

Example 4. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position P65.

Example 5. The polypeptide of example 4, wherein the substitution is selected from P65R, P65E, P65K, P65H, P65Y, P65Q, P65D, and P65N.

Example 6. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position H16.

Example 7. The polypeptide of example 6, wherein the substitution is selected from H16A, H16G, H16S, H16T, H16V, and H16P.

Example 8. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position D84.

Example 9. The polypeptide of example 8, wherein the substitution is selected from D84S, D84G, D84A, D84T, D84V, and D84P.

Example 10. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises substitutions at amino acid positions P65, H16, and D84.

Example 11. The polypeptide of example 10, wherein the modified IL-2 comprises substitutions P65R, H16A, and D84S.

Example 12. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position M23.

Example 13. The polypeptide of example 12, wherein the substitution is selected from M23A, M23G, M23S, M23T, M23V, and M23P.

Example 14. The polypeptide of example 13, wherein the modified IL-2 comprises substitutions P65R, H16A, D84S, and M23A.

Example 15. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position E95.

Example 16. The polypeptide of example 15, wherein the substitution is selected from E95Q, E95G, E95S, E95T, E95V, E95P, E95H, and E95N.

Example 17. The polypeptide of example 16, wherein the modified IL-2 comprises substitutions P65R, H16A, D84S, and E95Q.

Example 18. The polypeptide of example 17, wherein wherein the modified IL-2 comprises substitutions P65R, H16A, D84S, M23A, and E95Q.

Example 19. The polypeptide of any one of any one of the preceding examples, wherein the modified IL-2 comprises a substitution at amino acid position F42.

Example 20. The polypeptide of example 19, wherein the substitution at F42 is selected from F42K, F42A, F42R, F42G, F42S, and F42T.

Example 21. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises at least one substitution at at least one amino acid position selected from Y45 and L72.

Example 22. The polypeptide of example 21, wherein the modified IL-2 comprises at least one substitution selected from Y45A and L72G.

Example 23. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises at least one substitution at at least one amino acid position selected from T3 and C125.

Example 24. The polypeptide of example 23, wherein the modified IL-2 comprises at least one substitution selected from T3A, and C125A.

Example 25. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises a set of substitutions selected from H16A-F42K; D84S-F42K; E15S-F42K; M23A-F42K; E95Q-F42K; P65R-H16A; P65R-D84S; P65R-E15S; P65R-M23A; P65R-E95Q; T3A-C125S; T3A-P65R-C125S; T3A-H16A-C125S; T3A-D84S-C125S; T3A-H16A-P65R-C125S; T3A-P65R-D84S-C125S; T3A-H16A-P65R-D84S-C125S; T3A-H16A-M23A-P65R-D84S-C125S; T3A-H16A-P65R-D84S-E95Q-C125S, and T3A-H16A-M23A-P65R-D84S-E95Q-C125S.

Example 26. The polypeptide of example 25, wherein the modified IL-2 comprises the set of substitutions, and does not comprise any additional substitutions.

Example 27. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 84.

Example 28. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31.

Example 29. The polypeptide of any one of the preceding examples, wherein the modified IL-2 comprises an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31.

Example 30. The polypeptide of any one of the preceding examples, wherein the polypeptide comprises an Fc region.

Example 31. The polypeptide of example 30, wherein the modified IL-2 is fused to the N-terminus or the C-terminus of the Fc region.

Example 32. The polypeptide of example 30 or example 31, wherein the Fc region comprises a substitution at Kabat amino acid position T366.

Example 33. The polypeptide of example 32, wherein the Fc region comprises a T366W substitution.

Example 34. The polypeptide of example 31, wherein the Fc region comprises at least one substitution at at least one Kabat amino acid position selected from T366, L368, and Y407.

Example 35. The polypeptide of example 34, wherein the Fc region comprises T366S, L368A, and Y407V mutations.

Example 36. The polypeptide of any one of examples 30-35, wherein the Fc region comprises a substitution at a Kabat position selected from S354 and Y349.

Example 37. The polypeptide of example 36, wherein the Fc region comprises a S354C or a Y349C substitution.

Example 38. The polypeptide of any one of examples 30-37, wherein the Fc region comprises a substitution at Kabat amino acid position H435.

Example 39. The polypeptide of example 38, wherein the Fc region comprises a substitution selected from H435R and H435K.

Example 40. The polypeptide of any one of examples 30-39, wherein the Fc region comprises at least one substitution at at least one Kabat amino acid position selected from M252 and M428.

Example 41. The polypeptide of example 40, wherein the Fc region comprises M252Y and M428V substitutions.

Example 42. The polypeptide of any one of examples 30-41, wherein the Fc region comprises a deletion of Kabat amino acids E233, L234, and L235.

Example 43. The polypeptide of any one of examples 30-41, wherein the Fc region comprises at least one substitution at at least one amino acid position selected from L234, L235, and P329.

Example 44. The polypeptide of example 43, wherein the Fc region comprises L234A, L235A, and P329G substitutions.

Example 45. The polypeptide of any one of examples 30-44, wherein the Fc region comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 47-83.

Example 46. The polypeptide of any one of examples 30-44, wherein the Fc region is part of a heavy chain constant region.

Example 47. The polypeptide of example 46, wherein the heavy chain constant region is an IgG constant region.

Example 48. The polypeptide of example 47, wherein the heavy chain constant region is an IgG1, IgG2, IgG3, or IgG4 constant region.

Example 49. The polypeptide of any one of examples 30-48, wherein the modified IL-2 is fused to the C-terminus of the Fc region or heavy chain constant region.

Example 50. The polypeptide of example 49, wherein the modified IL-2 is fused to the C-terminus of the Fc region or heavy chain constant region via a linker comprising 1-20 amino acids.

Example 51. The polypeptide of example 50, wherein the linker comprises glycine amino acids.

Example 52. The polypeptide of example 51, wherein the linker comprises glycine and serine amino acids.

Example 53. The polypeptide of any one of examples 50-52, wherein a majority, or all, of the amino acids in the linker are glycine and serine.

Example 54. The polypeptide of any one of examples 30-33, 42, and 49-53, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 86, 87, 102, 103, or 104.

Example 55. The polypeptide of any one of the preceding examples, wherein the polypeptide comprises at least one antigen binding domain.

Example 56. The polypeptide of example 55, wherein the polypeptide comprises two, three, or four antigen binding domains.

Example 57. The polypeptide of example 55 or example 56, wherein at least one antigen binding domain specifically binds to a T-cell antigen or a natural killer cell antigen.

Example 58. The polypeptide of any one of examples 55-57, wherein at least one antigen binding domain specifically binds to a CD4⁺ T-cell antigen or a CD8⁺ T-cell antigen.

Example 59. The polypeptide of example 58, wherein the at least one antigen binding domain specifically binds to an antigen on an activated CD4⁺ T-cell or an activated CD8⁺ T-cell.

Example 60. The polypeptide of any one of examples 55-59, wherein at least one antigen binding domain is an agonist.

Example 61. The polypeptide of any one of examples 55-59, wherein the antigen binding domain is an antagonist.

Example 62. The polypeptide of any one of examples 55-61, wherein at least one antigen binding domain specifically binds to PD-1, CTLA-4, LAG3, TIM3, 4-1BB, OX40, GITR, CD8a, CD8b, CD4, NKp30, NKG2A, TIGIT, TGFβR1, TGFβR2, Fas, NKG2D, NKp46, PD-L1, CD107a, ICOS, TNFR2, or CD16a.

Example 63. The polypeptide of any one of examples 55-62, wherein at least one antigen binding domain specifically binds to PD-1.

Example 64. The polypeptide of any one of examples 55-63, wherein at least one antigen binding domain is a human or humanized antigen binding domain.

Example 65. The polypeptide of example 64, wherein each antigen binding domain is, independently, a human or humanized antigen binding domain.

Example 66. The polypeptide of any one of examples 55-65, wherein at least one antigen binding domain comprises a VHH domain.

Example 67. The polypeptide of example 66, wherein each antigen binding domain comprises a VHH domain.

Example 68. The polypeptide of any one of examples 55-65, wherein at least one antigen binding domain comprises a VH domain and a VL domain.

Example 69. The polypeptide of example 68, wherein at least one antigen binding domain comprises the VH domain and the VL domain of an antibody selected from pembrolizumab, nivolumab, AMP-514, TSR-042, STI-A1110, ipilimumab, tremelimumab, urelumab, utomilumab, atezolizumab, and durvalumab.

Example 70. The polypeptide of example 68 or 69, wherein the at least one antigen binding domain comprises a single chain Fv (scFv).

Example 71. The polypeptide of example 68 or 69, wherein the polypeptide comprises a heavy chain constant region, wherein the VH domain is fused to the heavy chain constant region, and wherein the VL domain is associated with the VH domain.

Example 72. The polypeptide of example 71, wherein the VL domain is fused to a light chain constant region.

Example 73. The polypeptide of example 72, wherein the light chain constant region is selected from kappa and lambda.

Example 74. The polypeptide of any one of examples 55-73, wherein each of the antigen binding domains are the same.

Example 75. The polypeptide of example 55-74, wherein each of the antigen binding domains specifically bind to the same antigen.

Example 76. The polypeptide of example 55-73, wherein at least one of the antigen binding domains specifically binds to a different antigen than at least one of the other antigen binding domains.

Example 77. The polypeptide of any one of examples 55-73, wherein at least one antigen binding domain specifically binds to PD-1 and at least one other antigen binding domain specifically binds to a T-cell antigen or natural killer cell antigen other than PD-1.

Example 78. The polypeptide of any one of examples 55-77, wherein at least one antigen binding domain binds to PD-1, CTLA-4, LAG3, TIM3, 4-1BB, OX40, GITR, CD8a, CD8b, CD4, NKp30, NKG2A, TIGIT, TGF β R1, TGF β R2, Fas, NKG2D, NKp46, PD-L1, CD107a, ICOS, TNFR2, or CD16a.

Example 79. The polypeptide of any one of examples 31-78, wherein the polypeptide forms a homodimer under physiological conditions.

Example 80. The polypeptide of any one of examples 1-79, wherein the modified IL-2 binds a human IL-2R with an affinity at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than the affinity of human wild type IL-2 for the IL-2R.

Example 81. A complex comprising a first polypeptide and a second polypeptide, wherein the first polypeptide is the polypeptide of any one of examples 1-79.

Example 82. The complex of example 81, wherein the first polypeptide comprises a first Fc region and the second polypeptide comprises a second Fc region.

- Example 83. The complex of example 81 or example 82, wherein each Fc region is an isotype selected from human IgG1, IgG2, IgG3, and IgG4.
- Example 84. The complex of example 83, wherein each Fc region is a human IgG1.
- Example 85. The complex of any one of examples 81-84, wherein each Fc region comprises a deletion of amino acids E233, L234, and L235.
- Example 86. The complex of any one of examples 81-85, wherein each Fc region comprises a H435R or H435K mutation.
- Example 87. The complex of any one of examples 81-86, wherein the Fc region comprises a mutations M252Y and M428L or mutations M252Y and M428V.
- Example 88. The complex of any one of examples 81-87, wherein the first Fc region or the second Fc region comprises a T366W mutation, and the other Fc region comprises mutations T366S, L368A, and Y407V.
- Example 89. The complex of example 88, wherein the first Fc region or the second Fc region comprises a S354C mutation.
- Example 90. The complex of any one of examples 81-89, wherein each Fc region independently comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 47-83.
- Example 91. The complex of any one of examples 81-90, wherein the second polypeptide does not comprise a modified IL2.
- Example 92. The complex of any one of examples 81-91, wherein the first polypeptide comprises at least one antigen binding domain.
- Example 93. The complex of any one of examples 81-92, wherein the second polypeptide comprises at least one antigen binding domain.
- Example 94. The complex of any one of examples 81-93, wherein the first polypeptide comprises a first antigen binding domain, an Fc region, and a modified IL-2.
- Example 95. The complex of example 94, wherein the first antigen binding domain is fused to the N-terminus of the Fc region and the modified IL-2 is fused to the C-terminus of the Fc region.
- Example 96. The complex of example 94 or example 95, wherein the second polypeptide comprises a second antigen binding domain and an Fc region.
- Example 97. The complex of example 96, wherein the first antigen binding domain and the second antigen binding domain are the same or different.
- Example 98. The complex of example 97, wherein:

- a) the first antigen binding domain and the second antigen binding domain both bind PD-1;
- b) the first antigen binding domain binds PD-1, and the second antigen binding domain binds LAG3;
- c) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CTLA-4;
- d) the first antigen binding domain binds PD-1, and the second antigen binding domain binds 4-1BB;
- e) the first antigen binding domain binds PD-1, and the second antigen binding domain binds OX40;
- f) the first antigen binding domain binds PD-1, and the second antigen binding domain binds GITR;
- g) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD8a;
- h) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD8b;
- i) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD4;
- j) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKp30;
- k) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKG2A;
- l) the first antigen binding domain binds PD-1, and the second antigen binding domain binds TIGIT;
- m) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKG2D;
- n) the first antigen binding domain binds PD-1, and the second antigen binding domain binds TGFBR2;
- o) the first antigen binding domain binds PD-1, and the second antigen binding domain binds Fas;
- p) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD107a;
- q) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKp46;

- r) the first antigen binding domain binds CD8a, and the second antigen binding domain binds TGFR β R2;
- s) the first antigen binding domain binds CD8a, and the second antigen binding domain binds Fas;
- t) the first antigen binding domain binds NKG2D, and the second antigen binding domain binds TGFR β R2;
- u) the first antigen binding domain binds NKG2D, and the second antigen binding domain binds Fas;
- v) the first antigen binding domain binds NKG2A, and the second antigen binding domain binds TGFR β R2;
- w) the first antigen binding domain binds NKG2A, and the second antigen binding domain binds Fas;
- x) the first antigen binding domain binds NKp46, and the second antigen binding domain binds TGFR β R2;
- y) the first antigen binding domain binds NKp46, and the second antigen binding domain binds Fas;
- z) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds LAG3;
- aa) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds Tim3;
- bb) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds OX40;
- cc) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds GITR;
- dd) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds CD107a;
- ee) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds NKp46; or
- ff) the first antigen binding domain binds ICOS, and the second antigen binding domain binds TNFR2.

Example 99. The complex of any one of examples 81-98, wherein the modified IL-2 binds a human IL-2R with an affinity at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than the affinity of human wild type IL-2 for the IL-2R.

Example 100. A pharmaceutical composition comprising a polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99 and a pharmaceutically acceptable carrier.

Example 101. An isolated nucleic acid the encodes a polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99.

Example 102. An expression vector comprising the nucleic acid of example 101.

Example 103. An isolated host cell comprising the nucleic acid of example 101 or the expression vector of example 102.

Example 104. An isolated host cell that expresses the polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99.

Example 105. A method of producing the polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99 comprising incubating the host cell of example 103 or example 104 under conditions suitable to express the polypeptide or complex.

Example 106. The method of example 105, further comprising isolating the polypeptide or complex.

Example 107. A method of increasing CD4⁺ and/or CD8⁺ T cell proliferation comprising contacting T cells with the polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99.

Example 108. The method of example 107, wherein the CD4⁺ and/or CD8⁺ T cells are in vitro.

Example 109. The method of example 107, wherein the CD4⁺ and/or CD8⁺ T cells are in vivo.

Example 110. The method of any one of examples 107-109, wherein the increase is at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold.

Example 111. A method of increasing NK cell proliferation comprising contacting NK cells with the polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99.

Example 112. The method of example 111, wherein the increase is at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold.

Example 113. A method of treating cancer comprising administering to a subject with cancer a pharmaceutically effective amount of the polypeptide of any one of examples 1-80 or the complex of any one of examples 81-99, or the pharmaceutical composition of example 100.

Example 114. The method of example 113, wherein the cancer is selected from basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; gastrointestinal cancer; glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; liver cancer; lung cancer; small-cell lung cancer; non-small cell lung cancer; adenocarcinoma of the lung; squamous carcinoma of the lung; melanoma; myeloma; neuroblastoma; oral cavity cancer; ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma; Hodgkin's lymphoma; non-Hodgkin's lymphoma; B-cell lymphoma; low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; Waldenstrom's macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; and chronic myeloblastic leukemia.

Example 115. The method of example 113 or 114, further comprising administering an additional therapeutic agent.

Example 116. The method of example 115, wherein the additional therapeutic agent is an anti-cancer agent.

Example 117. The method of example 116, wherein the anti-cancer agent is selected from a chemotherapeutic agent, an anti-cancer biologic, radiation therapy, CAR-T therapy, and an oncolytic virus.

Example 118. The method of example 116 or example 117, wherein the additional therapeutic agent is an anti-cancer biologic.

Example 119. The method of example 118, wherein the anti-cancer biologic is an agent that inhibits PD-1 and/or PD-L1.

Example 120. The method of example 118, wherein the anti-cancer biologic is an agent that inhibits VISTA, gpNMB, B7H3, B7H4, HHLA2, CTLA4, or TIGIT.

Example 121. The method of any one of examples 116-120, wherein the anti-cancer agent is an antibody.

- Example 122. The method of example 118, wherein the anti-cancer biologic is a cytokine.
- Example 123. The method of example 116, wherein the anti-cancer agent is CAR-T therapy.
- Example 124. The method of example 116, wherein the anti-cancer agent is an oncolytic virus.
- Example 125. The method of any one of examples 113-124, further comprising tumor resection and/or radiation therapy.

BRIEF DESCRIPTION OF THE FIGURES

[0007] FIG. 1A-1H show schematics of various IL-2 fusion protein formats. FIG. 1A shows IL-2 linked to the N-terminus of a heterodimeric, knob-in-hole IgG1 Fc. FIG. 1B shows IL-2 linked to the C-terminus of a heterodimeric IgG1 Fc of a single domain antibody. FIG. 1C-1E show IL-2 linked to one VHH (FIG. 1E), two identical VHHs (FIG. 1C), or two different VHHs (FIG. 1D). FIG. 1F shows IL-2 linked to the C-terminus of a homodimeric heavy chain constant region of a conventional antibody. FIG. 1G shows IL-2 linked to the C-terminus of a heterodimeric heavy chain constant region of a conventional antibody. FIG. 1H shows IL-2 fused to the C-terminus of a heterodimeric scFv antibody.

[0008] FIG. 2A-2C show binding of IL-2 fusion proteins comprising wild type IL-2 (FIG. 2A) or a modified IL-2 (FIG. 2A-2C) fused to the N-terminus of a heterodimeric Fc, as shown in FIG. 1A, to 293F cells transiently transfected with various combinations of the IL-2 receptor (CD25, CD122, and CD132), as measured by flow cytometry. “UT 293F” indicates untransfected 293F cells.

[0009] FIG. 3A-3B show binding of fusion proteins comprising wild type IL-2 or a modified IL-2 fused to the N-terminus of a heterodimeric Fc, as shown in FIG. 1A, to 293F cells transiently transfected with CD25 and CD122, as measured by flow cytometry.

[0010] FIG. 4A-4B show binding of fusion proteins comprising wild type IL-2 or a modified IL-2 fused to the N-terminus of a heterodimeric Fc, as shown in FIG. 1A, to 293F cells transiently transfected with CD122 and CD132; or CD25, CD122, and CD132, as measured by flow cytometry.

[0011] FIG. 5A-5B show binding of fusion proteins comprising wild type IL-2 or a modified IL-2 fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B, to resting and activated CD4⁺ T cells, as measured by flow cytometry. “Isotype control” indicates a control protein that does not comprise IL-2.

[0012] FIG. 6A-6B show binding of fusion proteins comprising wild type IL-2 or a modified IL-2 fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B, to enriched regulatory T cells (Tregs, FIG. 6A), induced regulatory T cells (induced Tregs, FIG. 6B), and enriched responder CD4⁺ T cells (Tresps, FIG. 6C), as measured by flow cytometry.

[0013] FIG. 7A-7D show the activity of fusion proteins comprising wild type IL-2 or a modified IL-2 fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B, on resting CD4⁺ and CD8⁺ T cells. Proliferation (FIG. 7A and 7C) and CD71 levels (FIG. 7B and 7D) were measured. FIG. 7E-7F show activity of wild type IL-2 or a modified IL-2 fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B, on resting CD4⁺ and CD8⁺ T cells as measured by flow cytometric detection of intracellular phosphorylated STAT5 levels. "Isotype" indicates a control protein that does not comprise IL-2.

[0014] FIG. 8A-8B show the proliferation and CD25 levels as a marker of activation of enriched Tregs following treatment for 7 days with a fusion protein comprising wild type IL-2 or a modified IL-2 fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B.

[0015] FIG. 9A-9D show activity and binding of pembrolizumab, an analog of pembrolizumab with IL-2-RAS fused to the heavy chain C-terminus, as shown in FIG. 1F, and IL-2-RAS alone (FIG. 9C and 9D) on CD8⁺ and CD4⁺ T cells. Activity on CD8⁺ (FIG. 9A) and CD4⁺ (FIG. 9B) T-cells was measured by flow cytometric detection of CellTrace™ Violet. Extent of binding to CD8⁺ T cells (FIG. 9C) and CD4⁺ T cells (FIG. 9D) was measured by flow cytometry.

[0016] FIG. 10A-10D show dependency of induction of CD8⁺ and CD4⁺ T cell proliferation on IL-2. Effects of pembrolizumab, non-targeted IL-2-RAS, and an analog of pembrolizumab with IL-2-RAS fused to the heavy chain C-terminus, as shown in FIG. 1F, on CD8⁺ (FIG. 10A and 10C) or CD4⁺ (FIG. 10B and 10D) T cell proliferation without pre-blocking (FIG. 10A and 10B) or pre-blocked with a saturating concentration of pembrolizumab (FIG. 10C and 10D) are shown.

[0017] FIG. 11 shows the recovery of CD4⁺ T responder (Tresp) cell proliferation by an analog of pembrolizumab with IL-2-RAS fused to the heavy chain C-terminus, as shown in FIG. 1F, as well as IL-2-RAS fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B and wild type IL-2 fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B. Tresp proliferation was induced by CD3 engagement (Tresp + beads), then suppressed using autologous regulatory T cells (Treg). "Tresp + beads" line shows baseline Tresp cell

proliferation with CD3 engagement in the absence of Treg cells. “No Ab” line shows baseline Tresp cell proliferation with CD3 engagement in the presence of Treg cells.

[0018] FIG. 12A-12B show the trans-activation of T cells by plate-bound non-targeted wild type IL-2 (“IL-2 WT”) or IL-2-RAS fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B. T cell activation was measured by flow cytometric detection of intracellular phosphorylated STAT5 levels. CD8⁺ T cell (FIG. 12A) and CD4⁺ T cell (FIG. 12B) responses are shown.

[0019] FIG. 13A-13I show activity and binding of IL-2-RAS fused to the C-terminus of a heterodimeric scFv antibody targeting NKp46, as shown in FIG. 1H, the heterodimeric scFv antibody targeting NKp46 alone, and fusion proteins comprising wild type IL-2 or IL-2-RAS fused to the C-terminus of a non-targeting VHH linked to a heterodimeric Fc, as shown in FIG. 1B, on NK cells, CD8⁺ T cells, and CD4⁺ T cells. Proliferation of NK cells (FIG. 13A), CD8⁺ T cells (FIG. 13B), and CD4⁺ T cells (FIG. 13C) and pSTAT levels of NK cells (FIG. 13D), CD8⁺ T cells (FIG. 13E), and CD4⁺ T cells (FIG. 13F) were measured by flow cytometry. Binding of the indicated polypeptides to NK cells (FIG. 13G), CD8⁺ T cells (FIG. 13H) and CD4⁺ T cells (FIG. 13I) was also measured by flow cytometry.

[0020] FIG. 14A-14H show activity and binding on CD8⁺ or CD4⁺ T cells of IL-2-RAS fused to the C-terminus of an anti-LAG3 heterodimeric conventional antibody (MAb), as shown in FIG. 1G, IL-2-RAS fused to the C-terminus of an anti-LAG3 VHH with a heterodimeric Fc, as shown in FIG. 1B, IL-2-RAS fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B, wild type IL-2 fused to the C-terminus of a non-targeted heterodimeric Fc, as shown in FIG. 1B, or LAG3-targeted MAb or LAG3-targeted VHH-Fc molecules without IL-2. Proliferation of CD8⁺ T cells (FIG. 14A) and CD4⁺ T cells (FIG. 14B) and expression of activation markers CD25 (FIG. 14C and 14D) and CD71 (FIG. 14E and 14F) on CD8⁺ T cells (FIG. 14C and 14E) and CD4⁺ T cells (FIG. 14D and 14F) were measured by flow cytometry. FIG. 14G and 14H show binding to pre-activated CD8⁺ T cells (FIG. 14G) and CD4⁺ T cells (FIG. 14H).

[0021] FIG. 15 shows activity of fusion proteins comprising the indicated modified IL-2 fused to the C-terminus of a VHH with a heterodimeric Fc, as shown in FIG. 1B, on HEK-Blue IL-2 reporter cells that do not express the VHH’s target antigen and therefore rely solely on binding of the modified IL-2 to the overexpressed IL-2 receptor for induction of the reporter gene. The activity of secreted embryonic alkaline phosphatase expressed in response to IL-2 receptor-mediated induction of pSTAT5 signaling in the reporter cell was measured.

DETAILED DESCRIPTION

[0022] Embodiments provided herein relate to polypeptides comprising a modified IL-2 that modulates the activity of T cells and their use in various methods of treating cancer.

Definitions and Various Embodiments

[0023] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0024] All references cited herein, including patent applications, patent publications, and Genbank Accession numbers are herein incorporated by reference, as if each individual reference were specifically and individually indicated to be incorporated by reference in its entirety.

[0025] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, *et al. eds.*, (2003)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor *eds.* (1995)), Harlow and Lane, *eds.* (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, *ed.* (1987)); Oligonucleotide Synthesis (M. J. Gait, *ed.*, 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J. E. Cellis, *ed.*, 1998) Academic Press; Animal Cell Culture (R. I. Freshney, *ed.*, 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, *eds.*, 1993-8) J. Wiley and Sons; Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, *eds.*); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, *eds.*, 1987); PCR: The Polymerase Chain Reaction, (Mullis *et al.*, *eds.*, 1994); Current Protocols in Immunology (J. E. Coligan *et al.*, *eds.*, 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: A Practical Approach (D. Catty, *ed.*, IRL Press, 1988-1989); Monoclonal Antibodies: A Practical Approach (P. Shepherd and C. Dean, *eds.*, Oxford University Press, 2000); Using Antibodies: A Laboratory Manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, *eds.*, Harwood Academic Publishers, 1995); and Cancer: Principles and Practice of Oncology (V. T. DeVita *et al.*, *eds.*, J.B. Lippincott Company, 1993); and updated versions thereof.

[0026] Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context or expressly indicated, singular terms shall include pluralities and plural terms shall include the singular. For any conflict in definitions between various sources or references, the definition provided herein will control.

[0027] In general, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). The “EU index as in Kabat” refers to the residue numbering of the human IgG1 EU antibody.

[0028] It is understood that embodiments of the invention described herein include “consisting” and/or “consisting essentially of” embodiments. As used herein, the singular form “a”, “an”, and “the” includes plural references unless indicated otherwise. Use of the term “or” herein is not meant to imply that alternatives are mutually exclusive.

[0029] In this application, the use of “or” means “and/or” unless expressly stated or understood by one skilled in the art. In the context of a multiple dependent claim, the use of “or” refers back to more than one preceding independent or dependent claim.

[0030] The phrase “reference sample”, “reference cell”, or “reference tissue”, denote a sample with at least one known characteristic that can be used as a comparison to a sample with at least one unknown characteristic. In some embodiments, a reference sample can be used as a positive or negative indicator. A reference sample can be used to establish a level of protein and/or mRNA that is present in, for example, healthy tissue, in contrast to a level of protein and/or mRNA present in the sample with unknown characteristics. In some embodiments, the reference sample comes from the same subject, but is from a different part of the subject than that being tested. In some embodiments, the reference sample is from a tissue area surrounding or adjacent to the cancer. In some embodiments, the reference sample is not from the subject being tested, but is a sample from a subject known to have, or not to have, a disorder in question (for example, a particular cancer or T cell related disorder). In some embodiments, the reference sample is from the same subject, but from a point in time before the subject developed cancer. In some embodiments, the reference sample is from a benign cancer sample, from the same or a different subject. When a negative reference sample is used for comparison, the level of expression or amount of the molecule in question in the negative reference sample will indicate a level at which one of skill in the art will appreciate, given the present disclosure, that there is no and/or a low level of the molecule. When a positive reference sample is used for comparison, the level of expression or amount of the molecule in question in the positive reference sample

will indicate a level at which one of skill in the art will appreciate, given the present disclosure, that there is a level of the molecule.

[0031] The terms “benefit”, “clinical benefit”, “responsiveness”, and “therapeutic responsiveness” as used herein in the context of benefiting from or responding to administration of a therapeutic agent, can be measured by assessing various endpoints, *e.g.*, inhibition, to some extent, of disease progression, including slowing down and complete arrest; reduction in the number of disease episodes and/or symptoms; reduction in lesion size; inhibition (that is, reduction, slowing down or complete stopping) of disease cell infiltration into adjacent peripheral organs and/or tissues; inhibition (that is, reduction, slowing down or complete stopping) of disease spread; relief, to some extent, of one or more symptoms associated with the disorder; increase in the length of disease-free presentation following treatment, for example, progression-free survival; increased overall survival; higher response rate; and/or decreased mortality at a given point of time following treatment. A subject or cancer that is “non-responsive” or “fails to respond” is one that has failed to meet the above noted qualifications to be “responsive”.

[0032] The terms “nucleic acid molecule”, “nucleic acid” and “polynucleotide” may be used interchangeably, and refer to a polymer of nucleotides. Such polymers of nucleotides may contain natural and/or non-natural nucleotides, and include, but are not limited to, DNA, RNA, and PNA. “Nucleic acid sequence” refers to the linear sequence of nucleotides comprised in the nucleic acid molecule or polynucleotide.

[0033] The terms “polypeptide” and “protein” are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present disclosure, a “polypeptide” refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification. “Amino acid sequence” refers to the linear sequence of amino acids comprised in a polypeptide or protein.

[0034] “IL-2” or “Interleukin-2” as used herein refers to any native, mature IL-2 that results from processing of an IL-2 precursor in a cell. The term includes IL-2 from any vertebrate

source, including mammals such as primates (*e.g.*, humans and cynomolgus or rhesus monkeys) and rodents (*e.g.*, mice and rats), unless otherwise indicated. The term also includes naturally-occurring variants of IL-2, such as splice variants or allelic variants. A nonlimiting exemplary human IL-2 amino acid sequence is shown, *e.g.*, in GenBank Accession No. NP_000577.2. *See* SEQ ID NO. 1 (mature form).

[0035] “Modified IL-2” as used herein refers to a polypeptide that differs from a wild type IL-2 amino acid sequence by a substitution at at least one amino acid position.

[0036] The term “specifically binds” to an antigen or epitope is a term that is well understood in the art, and methods to determine such specific binding are also well known in the art. A molecule is said to exhibit “specific binding” or “preferential binding” if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. An antigen binding domain “specifically binds” or “preferentially binds” to an antigen if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, a sdAb or VHH-containing polypeptide that specifically or preferentially binds to an epitope is a sdAb or VHH-containing polypeptide that binds this epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other epitopes on the same target antigen or epitopes on other target antigens. It is also understood by reading this definition that; for example, an antigen binding domain that specifically or preferentially binds to a first antigen may or may not specifically or preferentially bind to a second antigen. As such, “specific binding” or “preferential binding” does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding. “Specificity” refers to the ability of a binding protein to selectively bind an antigen.

[0037] As used herein, the term “modulate” with regard to the activity of IL-2 refers to a change in the activity of IL-2. In some embodiments, “modulate” refers to an increase in IL-2 activity.

[0038] As used herein, the term “epitope” refers to a site on a target molecule (for example, an antigen, such as a protein, nucleic acid, carbohydrate or lipid) to which an antigen binding molecule (for example, an antigen binding domain-containing polypeptide) binds. Epitopes often include a chemically active surface grouping of molecules such as amino acids, polypeptides or sugar side chains and have specific three-dimensional structural characteristics as well as specific charge characteristics. Epitopes can be formed both from contiguous and/or juxtaposed noncontiguous residues (for example, amino acids, nucleotides, sugars, lipid moiety) of the target molecule. Epitopes formed from contiguous residues (for example, amino acids, nucleotides, sugars, lipid moiety) typically are retained on exposure to denaturing solvents

whereas epitopes formed by tertiary folding typically are lost on treatment with denaturing solvents. An epitope may include but is not limited to at least 3, at least 5 or 8-10 residues (for example, amino acids or nucleotides). In some embodiments, an epitope is less than 20 residues (for example, amino acids or nucleotides) in length, less than 15 residues or less than 12 residues. Two antibodies may bind the same epitope within an antigen if they exhibit competitive binding for the antigen. In some embodiments, an epitope can be identified by a certain minimal distance to a CDR residue on the antigen binding molecule. In some embodiments, an epitope can be identified by the above distance, and further limited to those residues involved in a bond (for example, a hydrogen bond) between a residue of the antigen binding molecule and an antigen residue. An epitope can be identified by various scans as well, for example an alanine or arginine scan can indicate one or more residues that the antigen binding molecule can interact with. Unless explicitly denoted, a set of residues as an epitope does not exclude other residues from being part of the epitope for a particular antigen binding domain or molecule. Rather, the presence of such a set designates a minimal series (or set of species) of epitopes. Thus, in some embodiments, a set of residues identified as an epitope designates a minimal epitope of relevance for the antigen, rather than an exclusive list of residues for an epitope on an antigen.

[0039] A “nonlinear epitope” or “conformational epitope” comprises noncontiguous polypeptides, amino acids and/or sugars within the antigenic protein to which an antigen binding molecule (for example, an antigen binding domain-containing polypeptide) specific to the epitope binds. In some embodiments, at least one of the residues will be noncontiguous with the other noted residues of the epitope; however, one or more of the residues can also be contiguous with the other residues.

[0040] A “linear epitope” comprises contiguous polypeptides, amino acids and/or sugars within the antigenic protein to which an antigen-binding molecule (for example, an antigen binding domain-containing polypeptide) specific to the epitope binds. It is noted that, in some embodiments, not every one of the residues within the linear epitope need be directly bound (or involved in a bond) by the antigen binding molecule. In some embodiments, linear epitopes can be from immunizations with a peptide that effectively consisted of the sequence of the linear epitope, or from structural sections of a protein that are relatively isolated from the remainder of the protein (such that the antigen binding molecule can interact, at least primarily), just with that sequence section.

[0041] The terms “antibody” and “antigen binding molecule” are used interchangeably in the broadest sense and encompass various polypeptides that comprise antigen binding domains, including but not limited to conventional antibodies (typically comprising at least one heavy

chain and at least one light chain), single-domain antibodies (sdAbs, comprising just one chain, which is typically similar to a heavy chain), VHH-containing polypeptides (polypeptides comprising at least one heavy chain only antibody variable domain, or VHH), and fragments of any of the foregoing so long as they exhibit the desired antigen binding activity. In some embodiments, an antibody comprises a dimerization domain. Such dimerization domains include, but are not limited to, heavy chain constant domains (comprising CH1, hinge, CH2, and CH3, where CH1 typically pairs with a light chain constant domain, CL, while the hinge mediates dimerization) and Fc regions (comprising hinge, CH2, and CH3, where the hinge mediates dimerization). The term antibody also includes, but is not limited to, chimeric antibodies, humanized antibodies, and antibodies of various species such as camelid (including llama), shark, mouse, human, cynomolgus monkey, *etc.*

[0042] The terms “single domain antibody” and “sdAb” are used interchangeably herein to refer to an antibody having a single, monomeric domain, typically a heavy chain (or VHH), without a light chain.

[0043] The term “VHH” or “VHH domain” or “VHH antigen binding domain” as used herein refers to the antigen binding portion of a single-domain antibody, such as a camelid antibody or shark antibody. In some embodiments, a VHH comprises three CDRs and four framework regions, designated FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. In some embodiments, a VHH may be truncated at the N-terminus or C-terminus such that it comprises only a partial FR1 and/or FR4, or lacks one or both of those framework regions, so long as the VHH substantially maintains antigen binding and specificity.

[0044] The term “VHH-containing polypeptide” refers to a polypeptide that comprises at least one VHH domain. In some embodiments, a VHH polypeptide comprises two, three, or four or more VHH domains, wherein each VHH domain may be the same or different. In some embodiments, a VHH-containing polypeptide comprises an Fc region. In some such embodiments, the VHH polypeptide may form a dimer. Nonlimiting structures of VHH-containing polypeptides include VHH₁-Fc, VHH₁-VHH₂-Fc, and VHH₁-VHH₂-VHH₃-Fc, wherein VHH₁, VHH₂, and VHH₃ may be the same or different. In some embodiments of such structures, one VHH may be connected to another VHH by a linker, or one VHH may be connected to the Fc by a linker. In some such embodiments, the linker comprises 1-20 amino acids, preferably 1-20 amino acids predominantly composed of glycine and, optionally, serine. In some embodiments, when a VHH-containing polypeptide comprises an Fc, it forms a dimer. Thus, the structure VHH₁-VHH₂-Fc, if it forms a dimer, is considered to be tetravalent (i.e., the dimer has four VHH domains). Similarly, the structure VHH₁-VHH₂-VHH₃-Fc, if it forms a dimer, is considered to be hexavalent (i.e., the dimer has six VHH domains).

[0045] The term “monoclonal antibody” refers to an antibody (including an sdAb or VHH-containing polypeptide) of a substantially homogeneous population of antibodies, that is, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Thus, a sample of monoclonal antibodies can bind to the same epitope on the antigen. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies may be made by the hybridoma method first described by Kohler and Milstein, 1975, *Nature* 256:495, or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described in McCafferty *et al.*, 1990, *Nature* 348:552-554, for example.

[0046] The term “CDR” denotes a complementarity determining region as defined by at least one manner of identification to one of skill in the art. In some embodiments, CDRs can be defined in accordance with any of the Chothia numbering schemes, the Kabat numbering scheme, a combination of Kabat and Chothia, the AbM definition, and/or the contact definition. A VHH comprises three CDRs, designated CDR1, CDR2, and CDR3.

[0047] The term “heavy chain constant region” as used herein refers to a region comprising at least three heavy chain constant domains, C_{H1} , hinge, C_{H2} , and C_{H3} . Of course, non-function-altering deletions and alterations within the domains are encompassed within the scope of the term “heavy chain constant region,” unless designated otherwise. Nonlimiting exemplary heavy chain constant regions include γ , δ , and α . Nonlimiting exemplary heavy chain constant regions also include ϵ and μ . Each heavy constant region corresponds to an antibody isotype. For example, an antibody comprising a γ constant region is an IgG antibody, an antibody comprising a δ constant region is an IgD antibody, and an antibody comprising an α constant region is an IgA antibody. Further, an antibody comprising a μ constant region is an IgM antibody, and an antibody comprising an ϵ constant region is an IgE antibody. Certain isotypes can be further subdivided into subclasses. For example, IgG antibodies include, but are not limited to, IgG1 (comprising a γ_1 constant region), IgG2 (comprising a γ_2 constant region), IgG3 (comprising a γ_3 constant region), and IgG4 (comprising a γ_4 constant region) antibodies; IgA antibodies include, but are not limited to, IgA1 (comprising an α_1 constant region) and IgA2 (comprising an α_2 constant region) antibodies; and IgM antibodies include, but are not limited to, IgM1 and IgM2.

[0048] A “Fc region” as used herein refers to a portion of a heavy chain constant region comprising CH2 and CH3. In some embodiments, an Fc region comprises a hinge, CH2, and CH3. In various embodiments, when an Fc region comprises a hinge, the hinge mediates dimerization between two Fc-containing polypeptides. An Fc region may be of any antibody heavy chain constant region isotype discussed herein. In some embodiments, an Fc region is an IgG1, IgG2, IgG3, or IgG4.

[0049] An “acceptor human framework” as used herein is a framework comprising the amino acid sequence of a heavy chain variable domain (V_H) framework derived from a human immunoglobulin framework or a human consensus framework, as discussed herein. An acceptor human framework derived from a human immunoglobulin framework or a human consensus framework can comprise the same amino acid sequence thereof, or it can contain amino acid sequence changes. In some embodiments, the number of amino acid changes are fewer than 10, or fewer than 9, or fewer than 8, or fewer than 7, or fewer than 6, or fewer than 5, or fewer than 4, or fewer than 3, across all of the human frameworks in a single antigen binding domain, such as a VHH.

[0050] “Affinity” refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (for example, an antibody or VHH-containing polypeptide) and its binding partner (for example, an antigen). The affinity or the apparent affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D) or the $K_{D\text{-apparent}}$, respectively. Affinity can be measured by common methods known in the art (such as, for example, ELISA K_D , KinExA, flow cytometry, and/or surface plasmon resonance devices), including those described herein. Such methods include, but are not limited to, methods involving BIAcore®, Octet®, or flow cytometry.

[0051] The term “ K_D ”, as used herein, refers to the equilibrium dissociation constant of an antigen binding molecule/antigen interaction. When the term “ K_D ” is used herein, it includes K_D and $K_{D\text{-apparent}}$.

[0052] In some embodiments, the K_D of the antigen binding molecule is measured by flow cytometry using an antigen-expressing cell line and fitting the mean fluorescence measured at each antibody concentration to a non-linear one-site binding equation (Prism Software graphpad). In some such embodiments, the K_D is $K_{D\text{-apparent}}$.

[0053] The term “biological activity” refers to any one or more biological properties of a molecule (whether present naturally as found in vivo, or provided or enabled by recombinant means). Biological properties include, but are not limited to, binding a ligand, inducing or increasing cell proliferation (such as T cell proliferation), and inducing or increasing expression of cytokines.

[0054] The term “IL-2 activity” or “biological activity” of IL-2, as used herein, includes any biological effect or at least one of the biologically relevant functions of IL-2. In some embodiments, IL-2 activity includes the ability of IL-2 to induce T cell proliferation and/or activate natural killer (NK) cells. Nonlimiting exemplary IL-2 activities include increasing pSTAT5 expression, increasing proliferation of CD4⁺ and/or CD8⁺ T cells, increasing CD71 expression on T cells, and reducing the suppressive activity of Treg cells on CD4⁺ and CD8⁺ T cell activation and proliferation.

[0055] An “agonist” or “activating” antibody (such as a sdAb or VHH-containing polypeptide) is one that increases and/or activates a biological activity of the target antigen. In some embodiments, the agonist antibody binds to an antigen and increases its biological activity by at least about 20%, 40%, 60%, 80%, 85% or more.

[0056] An “antagonist”, a “blocking” or “neutralizing” antibody is one that decreases and/or inactivates a biological activity of the target antigen. In some embodiments, the neutralizing antibody binds to an antigen and reduces its biological activity by at least about 20%, 40%, 60%, 80%, 85%, 90%, 95%, 99% or more.

[0057] An “affinity matured” VHH-containing polypeptide refers to a VHH-containing polypeptide with one or more alterations in one or more CDRs compared to a parent VHH-containing polypeptide that does not possess such alterations, such alterations resulting in an improvement in the affinity of the VHH-containing polypeptide for antigen.

[0058] A “humanized VHH” as used herein refers to a VHH in which one or more framework regions have been substantially replaced with human framework regions. In some instances, certain framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized VHH can comprise residues that are found neither in the original VHH nor in the human framework sequences, but are included to further refine and optimize VHH or VHH-containing polypeptide performance. In some embodiments, a humanized VHH-containing polypeptide comprises a human Fc region. As will be appreciated, a humanized sequence can be identified by its primary sequence and does not necessarily denote the process by which the antibody was created.

[0059] A “functional Fc region” possesses an “effector function” of a native sequence Fc region. Exemplary “effector functions” include Fc receptor binding; C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (for example B-cell receptor); and B-cell activation, etc. Such effector functions generally require the Fc region to be combined with a binding domain (for example, an antibody variable domain) and can be assessed using various assays.

[0060] A “native sequence Fc region” comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

[0061] A “variant Fc region” comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. In some embodiments, a “variant Fc region” comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, yet retains at least one effector function of the native sequence Fc region. In some embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, for example, from about one to about ten amino acid substitutions, and preferably, from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. In some embodiments, the variant Fc region herein will possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, at least about 90% sequence identity therewith, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity therewith.

[0062] “Fc receptor” or “FcR” describes a receptor that binds to the Fc region of an antibody. In some embodiments, an Fc γ R is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc γ RI, Fc γ RII, and Fc γ RIII subclasses, including allelic variants and alternatively spliced forms of those receptors. Fc γ RII receptors include Fc γ RIIA (an “activating receptor”) and Fc γ RIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc γ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc γ RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (See, for example, Daeron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas *et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein. For example, the term “Fc receptor” or “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, for example, Ghetie

and Ward, *Immunol. Today* 18(12):592-598 (1997); Ghetie *et al.*, *Nature Biotechnology*, 15(7):637-640 (1997); Hinton *et al.*, *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton *et al.*).

[0063] The term “substantially similar” or “substantially the same,” as used herein, denotes a sufficiently high degree of similarity between two or more numeric values such that one of skill in the art would consider the difference between the two or more values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said value. In some embodiments the two or more substantially similar values differ by no more than about any one of 5%, 10%, 15%, 20%, 25%, or 50%.

[0064] A polypeptide “variant” means a biologically active polypeptide having at least about 80% amino acid sequence identity with the native sequence polypeptide after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the polypeptide. In some embodiments, a variant will have at least about 80% amino acid sequence identity. In some embodiments, a variant will have at least about 90% amino acid sequence identity. In some embodiments, a variant will have at least about 95% amino acid sequence identity with the native sequence polypeptide.

[0065] As used herein, “percent (%) amino acid sequence identity” and “homology” with respect to a peptide, polypeptide or antibody sequence are defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific peptide or polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or MEGALIGN™ (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0066] An amino acid substitution may include but are not limited to the replacement of one amino acid in a polypeptide with another amino acid. Exemplary substitutions are shown in Table 1. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, for example, retained/improved antigen or receptor binding, reduced antigen or receptor binding, decreased immunogenicity, or improved ADCC or CDC.

Table 1

Original Residue	Exemplary Substitutions
Ala (A)	Val; Leu; Ile
Arg (R)	Lys; Gln; Asn
Asn (N)	Gln; His; Asp, Lys; Arg
Asp (D)	Glu; Asn
Cys (C)	Ser; Ala
Gln (Q)	Asn; Glu
Glu (E)	Asp; Gln
Gly (G)	Ala
His (H)	Asn; Gln; Lys; Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg; Gln; Asn
Met (M)	Leu; Phe; Ile
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala
Ser (S)	Thr
Thr (T)	Val; Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe; Thr; Ser
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine

[0067] Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

[0068] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0069] The term “vector” is used to describe a polynucleotide that can be engineered to contain a cloned polynucleotide or polynucleotides that can be propagated in a host cell. A vector can include one or more of the following elements: an origin of replication, one or more regulatory sequences (such as, for example, promoters and/or enhancers) that regulate the expression of the polypeptide of interest, and/or one or more selectable marker genes (such as, for example, antibiotic resistance genes and genes that can be used in colorimetric assays, for example, β -galactosidase). The term “expression vector” refers to a vector that is used to express a polypeptide of interest in a host cell.

[0070] A “host cell” refers to a cell that may be or has been a recipient of a vector or isolated polynucleotide. Host cells may be prokaryotic cells or eukaryotic cells. Exemplary eukaryotic cells include mammalian cells, such as primate or non-primate animal cells; fungal cells, such as yeast; plant cells; and insect cells. Nonlimiting exemplary mammalian cells include, but are not limited to, NSO cells, PER.C6[®] cells (Crucell), and 293F and CHO cells, and their derivatives, such as 293-6E, CHO-DG44, CHO-K1, CHO-S, and CHO-DS cells. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) as provided herein.

[0071] The term “isolated” as used herein refers to a molecule that has been separated from at least some of the components with which it is typically found in nature or produced. For example, a polypeptide is referred to as “isolated” when it is separated from at least some of the components of the cell in which it was produced. Where a polypeptide is secreted by a cell after expression, physically separating the supernatant containing the polypeptide from the cell that produced it is considered to be “isolating” the polypeptide. Similarly, a polynucleotide is referred to as “isolated” when it is not part of the larger polynucleotide (such as, for example, genomic DNA or mitochondrial DNA, in the case of a DNA polynucleotide) in which it is typically found in nature, or is separated from at least some of the components of the cell in

which it was produced, for example, in the case of an RNA polynucleotide. Thus, a DNA polynucleotide that is contained in a vector inside a host cell may be referred to as “isolated”.

[0072] The terms “individual” and “subject” are used interchangeably herein to refer to an animal; for example, a mammal. In some embodiments, methods of treating mammals, including, but not limited to, humans, rodents, simians, felines, canines, equines, bovines, porcines, ovines, caprines, mammalian laboratory animals, mammalian farm animals, mammalian sport animals, and mammalian pets, are provided. In some examples, an “individual” or “subject” refers to an individual or subject in need of treatment for a disease or disorder. In some embodiments, the subject to receive the treatment can be a patient, designating the fact that the subject has been identified as having a disorder of relevance to the treatment, or being at adequate risk of contracting the disorder.

[0073] A “disease” or “disorder” as used herein refers to a condition where treatment is needed and/or desired.

[0074] The term “tumor cell”, “cancer cell”, “cancer”, “tumor”, and/or “neoplasm”, unless otherwise designated, are used herein interchangeably and refer to a cell (or cells) exhibiting an uncontrolled growth and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of bodily organs and systems. Included in this definition are benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases.

[0075] The terms “cancer” and “tumor” encompass solid and hematological/lymphatic cancers and also encompass malignant, pre-malignant, and benign growth, such as dysplasia. Also, included in this definition are cells having abnormal proliferation that is not impeded (*e.g.* immune evasion and immune escape mechanisms) by the immune system (*e.g.* virus infected cells). Exemplary cancers include, but are not limited to: basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the

urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

[0076] The term “non-tumor cell” as used herein refers to a normal cells or tissue.

Exemplary non-tumor cells include, but are not limited to: T cells, B-cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, monocytes, macrophages, epithelial cells, fibroblasts, hepatocytes, interstitial kidney cells, fibroblast-like synoviocytes, osteoblasts, and cells located in the breast, skeletal muscle, pancreas, stomach, ovary, small intestines, placenta, uterus, testis, kidney, lung, heart, brain, liver, prostate, colon, lymphoid organs, bone, and bone-derived mesenchymal stem cells. The term “a cell or tissue located in the periphery” as used herein refers to non-tumor cells not located near tumor cells and/or within the tumor microenvironment.

[0077] The term “cells or tissue within the tumor microenvironment” as used herein refers to the cells, molecules, extracellular matrix and/or blood vessels that surround and/or feed a tumor cell. Exemplary cells or tissue within the tumor microenvironment include, but are not limited to: tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T cells; regulatory T cells (Treg cells); macrophages; neutrophils; myeloid-derived suppressor cells (MDSCs) and other immune cells located proximal to a tumor. Methods for identifying tumor cells, and/or cells/tissues located within the tumor microenvironment are well known in the art, as described herein, below.

[0078] In some embodiments, an “increase” or “decrease” refers to a statistically significant increase or decrease, respectively. As will be clear to the skilled person, “modulating” can also involve effecting a change (which can either be an increase or a decrease) in affinity, avidity, specificity and/or selectivity of a target or antigen, for one or more of its ligands, binding partners, partners for association into a homomultimeric or heteromultimeric form, or substrates; effecting a change (which can either be an increase or a decrease) in the sensitivity of the target

or antigen for one or more conditions in the medium or surroundings in which the target or antigen is present (such as pH, ion strength, the presence of co-factors, *etc.*); and/or cellular proliferation or cytokine production, compared to the same conditions but without the presence of a test agent. This can be determined in any suitable manner and/or using any suitable assay known *per se* or described herein, depending on the target involved.

[0079] As used herein, “an immune response” is meant to encompass cellular and/or humoral immune responses that are sufficient to inhibit or prevent onset or ameliorate the symptoms of disease (for example, cancer or cancer metastasis). “An immune response” can encompass aspects of both the innate and adaptive immune systems.

[0080] As used herein, “treatment” is an approach for obtaining beneficial or desired clinical results. “Treatment” as used herein, covers any administration or application of a therapeutic for disease in a mammal, including a human. For purposes of this disclosure, beneficial or desired clinical results include, but are not limited to, any one or more of: alleviation of one or more symptoms, diminishment of extent of disease, preventing or delaying spread (for example, metastasis, for example metastasis to the lung or to the lymph node) of disease, preventing or delaying recurrence of disease, delay or slowing of disease progression, amelioration of the disease state, inhibiting the disease or progression of the disease, inhibiting or slowing the disease or its progression, arresting its development, and remission (whether partial or total). Also encompassed by “treatment” is a reduction of pathological consequence of a proliferative disease. The methods provided herein contemplate any one or more of these aspects of treatment. In-line with the above, the term treatment does not require one-hundred percent removal of all aspects of the disorder.

[0081] “Ameliorating” means a lessening or improvement of one or more symptoms as compared to not administering a therapeutic agent. “Ameliorating” also includes shortening or reduction in duration of a symptom.

[0082] The term “anti-cancer agent” is used herein in its broadest sense to refer to agents that are used in the treatment of one or more cancers. Exemplary classes of such agents include, but are not limited to, chemotherapeutic agents, anti-cancer biologics (such as cytokines, receptor extracellular domain-Fc fusions, and antibodies), radiation therapy, CAR-T therapy, therapeutic oligonucleotides (such as antisense oligonucleotides and siRNAs) and oncolytic viruses.

[0083] The term “biological sample” means a quantity of a substance from a living thing or formerly living thing. Such substances include, but are not limited to, blood, (for example, whole blood), plasma, serum, urine, amniotic fluid, synovial fluid, endothelial cells, leukocytes, monocytes, other cells, organs, tissues, bone marrow, lymph nodes and spleen.

[0084] The term “control” or “reference” refers to a composition known to not contain an analyte (“negative control”) or to contain an analyte (“positive control”). A positive control can comprise a known concentration of analyte.

[0085] The terms “inhibition” or “inhibit” refer to a decrease or cessation of any phenotypic characteristic or to the decrease or cessation in the incidence, degree, or likelihood of that characteristic. To “reduce” or “inhibit” is to decrease, reduce or arrest an activity, function, and/or amount as compared to a reference. In some embodiments, by “reduce” or “inhibit” is meant the ability to cause an overall decrease of 10% or greater. In some embodiments, by “reduce” or “inhibit” is meant the ability to cause an overall decrease of 50% or greater. In some embodiments, by “reduce” or “inhibit” is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater. In some embodiments, the amount noted above is inhibited or decreased over a period of time, relative to a control over the same period of time.

[0086] As used herein, “delaying development of a disease” means to defer, hinder, slow, retard, stabilize, suppress and/or postpone development of the disease (such as cancer). This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease. For example, a late stage cancer, such as development of metastasis, may be delayed.

[0087] “Preventing,” as used herein, includes providing prophylaxis with respect to the occurrence or recurrence of a disease in a subject that may be predisposed to the disease but has not yet been diagnosed with the disease. Unless otherwise specified, the terms “reduce”, “inhibit”, or “prevent” do not denote or require complete prevention over all time, but just over the time period being measured.

[0088] A “therapeutically effective amount” of a substance/molecule, agonist or antagonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects. A therapeutically effective amount may be delivered in one or more administrations. A therapeutically effective amount refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic and/or prophylactic result.

[0089] The terms “pharmaceutical formulation” and “pharmaceutical composition” refer to a preparation which is in such form as to permit the biological activity of the active ingredient(s) to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations may be sterile.

[0090] A “pharmaceutically acceptable carrier” refers to a non-toxic solid, semisolid, or liquid filler, diluent, encapsulating material, formulation auxiliary, or carrier conventional in the art for use with a therapeutic agent that together comprise a “pharmaceutical composition” for administration to a subject. A pharmaceutically acceptable carrier is non-toxic to recipients at the dosages and concentrations employed and are compatible with other ingredients of the formulation. The pharmaceutically acceptable carrier is appropriate for the formulation employed.

[0091] Administration “in combination with” one or more further therapeutic agents includes simultaneous (concurrent) and sequential administration in any order.

[0092] The term “concurrently” is used herein to refer to administration of two or more therapeutic agents, where at least part of the administration overlaps in time, or where the administration of one therapeutic agent falls within a short period of time relative to administration of the other therapeutic agent, or wherein the therapeutic effects of both agents overlap for at least a period of time.

[0093] The term “sequentially” is used herein to refer to administration of two or more therapeutic agents that does not overlap in time, or wherein the therapeutic effects of the agents do not overlap.

[0094] As used herein, “in conjunction with” refers to administration of one treatment modality in addition to another treatment modality. As such, “in conjunction with” refers to administration of one treatment modality before, during, or after administration of the other treatment modality to the individual.

[0095] The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

[0096] An “article of manufacture” is any manufacture (for example, a package or container) or kit comprising at least one reagent, for example, a medicament for treatment of a disease or disorder (for example, cancer), or a probe for specifically detecting a biomarker described herein. In some embodiments, the manufacture or kit is promoted, distributed, or sold as a unit for performing the methods described herein.

[0097] The terms “label” and “detectable label” mean a moiety attached, for example, to an antibody or antigen to render a reaction (for example, binding) between the members of the specific binding pair, detectable. The labeled member of the specific binding pair is referred to as “detectably labeled.” Thus, the term “labeled binding protein” refers to a protein with a label incorporated that provides for the identification of the binding protein. In some embodiments,

the label is a detectable marker that can produce a signal that is detectable by visual or instrumental means, for example, incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (for example, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (for example, ^3H , ^{14}C , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I , ^{177}Lu , ^{166}Ho , or ^{153}Sm); chromogens, fluorescent labels (for example, FITC, rhodamine, lanthanide phosphors), enzymatic labels (for example, horseradish peroxidase, luciferase, alkaline phosphatase); chemiluminescent markers; biotinyl groups; predetermined polypeptide epitopes recognized by a secondary reporter (for example, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags); and magnetic agents, such as gadolinium chelates. Representative examples of labels commonly employed for immunoassays include moieties that produce light, for example, acridinium compounds, and moieties that produce fluorescence, for example, fluorescein. In this regard, the moiety itself may not be detectably labeled but may become detectable upon reaction with yet another moiety.

Exemplary modified IL-2-containing polypeptides

[0098] Polypeptides comprising a modified IL-2 are provided herein. In some embodiments, the modified IL-2 comprises at least one amino acid substitution that reduces the affinity of the modified IL-2 for an IL-2 receptor compared to a wild type IL-2. In various embodiments, the polypeptide comprising a modified IL-2 provided herein is an agonist of an IL-2R. In some embodiments, the modified IL-2 is a modified human IL-2, and the IL-2R is a human IL-2R. In some embodiments, the modified IL-2 binds a human IL-2R with an affinity at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than the affinity of human wild type IL-2 for the IL-2R.

[0099] In various embodiments, the polypeptides comprising a modified IL-2 comprise at least one antigen binding domain that binds a T cell or natural killer (NK) cell antigen. In some embodiments, a polypeptide comprising a modified IL-2 provided herein comprises one, two, three, four, five, six, seven, or eight antigen binding domains, wherein at least one, or all, bind a T cell or natural killer cell antigen. In some embodiments, a polypeptide comprising a modified IL-2 provided herein comprises one, two, three, or four antigen binding domains, wherein at least one, or all, bind a T cell or natural killer cell antigen. In some embodiments, the modified IL-2 containing polypeptide does not bind or activate IL-2R in the absence of an antigen binding domain. In some embodiments, the modified IL-2 containing polypeptide binds and/or activates

IL-2R on a cell only when the polypeptide comprises an antigen binding domain that is bound to an antigen on the same cell as the IL-2R.

[00100] In various embodiments, a modified IL-2 comprises at least one substitution at at least one amino acid position selected from P65, D84, E95, M23, and H16. In some embodiments, a modified IL-2 comprises substitutions at amino acid positions P65, H16, and D84. In some embodiments, a modified IL-2 comprises substitutions at amino acid positions P65, H16, D84, and M23. In some embodiments, a modified IL-2 comprises substitutions at amino acid positions P65, H16, D84, and E95. In some embodiments, a modified IL-2 comprises substitutions at amino acid positions P65, H16, D84, M23, and E95.

[00101] In some embodiments, the substitution at amino acid position P65 is selected from P65R, P65E, P65K, P65H, P65Y, P65Q, P65D, and P65N. In some embodiments, the substitution at amino acid position H16 is selected from H16A, H16G, H16S, H16T, H16V, and H16P. In some embodiments, the substitution at amino acid position D84 is selected from D84S, D84G, D84A, D84T, D84V, and D84P. In some embodiments, the substitution at amino acid position M23 is selected from M23A, M23G, M23S, M23T, M23V, and M23P. In some embodiments, the substitution at amino acid position E95 is selected from E95Q, E95G, E95S, E95T, E95V, E95P, E95H, and E95N.

[00102] In some embodiments, the modified IL-2 further comprises a substitution at amino acid position F42. In some such embodiments, the substitution at F42 is selected from F42K, F42A, F42R, F42A, F42G, F42S, and F42T.

[00103] In some embodiments, the modified IL-2 further comprises at least one substitution at at least one amino acid position selected from Y45 and L72. In some such embodiments, the modified IL-2 comprises at least one substitution selected from Y45A and L72G.

[00104] In some embodiments, the modified IL-2 further comprises at least one substitution at at least one amino acid position selected from T3 and C125. In some such embodiments, the modified IL-2 comprises at least one substitution selected from T3A, and C125A.

[00105] In some embodiments, the modified IL-2 comprises substitutions P65R, H16A, and D84S. In some embodiments, the modified IL-2 comprises substitutions P65R, H16A, D84S, and M23A. In some embodiments, the modified IL-2 comprises substitutions P65R, H16A, D84S, and E95Q. In some embodiments, the modified IL-2 comprises substitutions P65R, H16A, D84S, M23A, and E95Q. In some embodiments, the modified IL-2 comprises substitutions selected from H16A-F42K; D84S-F42K; E15S-F42K; M23A-F42K; E95Q-F42K; P65R-H16A; P65R-D84S; P65R-E15S; P65R-M23A; P65R-E95Q; T3A-C125S; T3A-P65R-

C125S; T3A-H16A-C125S; T3A-D84S-C125S; T3A-H16A-P65R-C125S; T3A-P65R-D84S-C125S; T3A-H16A-P65R-D84S-C125S; T3A-H16A-M23A-P65R-D84S-C125S; T3A-H16A-P65R-D84S-E95Q-C125S, and T3A-H16A-M23A-P65R-D84S-E95Q-C125S.

[00106] In any of the embodiments described herein, the modified IL-2 may be a modified human IL-2. In various embodiments, the amino acid positions of the substitutions correspond to the amino acid positions in SEQ ID NO: 1.

[00107] In some embodiments, the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 84, and including one or more of the substitutions discussed herein. In some embodiments, the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31, and including one or more of the substitutions discussed herein. In some embodiments, the modified IL-2 comprises an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31. In some embodiments, the modified IL-2 comprises an amino acid sequence selected from SEQ ID NOs: 3, 5-9, 12-21, and 23-31.

[00108] In some embodiments, a modified IL-2 containing polypeptide comprises at least one antigen binding domain that binds a T cell or natural killer cell antigen and an Fc region. In some embodiments, a modified IL-2 containing polypeptide provided herein comprises one, two, three, or four antigen binding domains and an Fc region. In some embodiments, an Fc region mediates dimerization of the modified IL-2 containing polypeptide at physiological conditions such that a dimer is formed that doubles the number of antigen binding sites. For example, a modified IL-2 containing polypeptide comprising three antigen binding domains and an Fc region is trivalent as a monomer, but at physiological conditions, the Fc region may mediate dimerization, such that the modified IL-2 containing polypeptide exists as a hexavalent dimer under such conditions.

[00109] In various embodiments, a polypeptide comprising a modified IL-2 comprises a sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31. In various embodiments, a polypeptide comprising a modified IL-2 comprises a sequence selected from SEQ ID NOs: 3, 5-9, and 12-21, and 23-31. In various embodiments, a polypeptide comprising a modified IL-2 comprises SEQ ID NO: 21. In some embodiments, the polypeptide further comprises an antigen binding domain. In some embodiments, the antigen binding domain is humanized.

[00110] In some embodiments, the at least one antigen binding domain is a natural or native cognate binding partner, an Anticalin (engineered lipocalin), a Darpin, a Fynomer, a Centyrin (engineered fibronectin III domain), a cystine-knot domain, an Affilin, an Affibody, or an engineered CH3 domain. In some embodiments, the natural cognate binding partner comprises

a ligand or an extracellular domain, or binding fragment thereof, of the native cognate binding partner of the tumor associated antigen (TAA), or a variant thereof that exhibits binding activity to the TAA.

[00111] In some embodiments, the polypeptide comprising the modified IL-2 and at least one antigen binding domain enhances anti-tumor T cell responses or natural killer cell responses while avoiding Tregs, peripheral T cells, and endothelial cells. In some such embodiments, the at least one antigen binding domain targets the modified IL-2 to activated T cells. In some embodiments, the modified IL-2 binds and modulates an IL-2R only when the IL-2R is on the same cell as the antigen bound by the at least one antigen binding domain. In some embodiments, the modified IL-2 does not bind or activate an IL-2R when the IL-2R is on a different cell than the cell expressing the antigen bound by the at least one antigen binding domain.

[00112] In various embodiments, the antigen-binding domain binds to a protein selected from PD-1, CTLA-4, LAG3, TIM3, 4-1BB, OX40, GITR, CD8a, CD8b, CD4, NKp30, NKG2A, TIGIT, TGF β R1, TGF β R2, Fas, NKG2D, NKp46, PD-L1, CD107a, ICOS, TNFR2, and CD16a. In some embodiments, the polypeptide comprising a modified IL-2 comprises an antigen-binding domain of nivolumab (BMS; PD-1); pembrolizumab (Merck; PD-1); AMP-514 (Amplimmune; PD-1); TSR-042 (Tesaro/AnaptysBio, ANB-011; PD-1); STI-A1110 (Sorrento Therapeutics; PD-1); ipilimumab (BMS; CTLA-4); tremelimumab (AstraZeneca, CTLA-4); urelumab (BMS, 4-1BB); utomilumab (Pfizer, 4-1BB); atezolizumab (Roche, PD-L1), durvalumab (AstraZeneca, PD-L1); monalizumab (NKG2A, Innate Pharma and AstraZeneca); BMS-986016 (Bristol-Myers Squibb, LAG-3).

[00113] In some embodiments, the polypeptide comprises at least one antigen binding domain that specifically binds to PD-1. In some embodiments, the polypeptide comprises at least one antigen binding domain that specifically binds to LAG3. In some embodiments, the polypeptide comprises at least one antigen binding domain that specifically binds to NKp46. In some embodiments, the polypeptide comprises at least one antigen binding domain that specifically binds to NKG2D. In some embodiments, the polypeptide comprises at least one antigen binding domain that specifically binds to CD8a.

[00114] In some embodiments, an antigen binding domain may be humanized. Polypeptides comprising humanized antigen binding domains (such as VHH-containing polypeptides) are useful as therapeutic molecules because humanized antigen binding domains and humanized antibodies reduce or eliminate the human immune response to non-human antibodies, which can result in an immune response to an antibody therapeutic, and decreased effectiveness of the therapeutic. Generally, a humanized antigen binding domain or humanized

antibody comprises one or more variable domains in which CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antigen binding domain or humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antigen binding domain or humanized antibody are substituted with corresponding residues from a non-human antibody (for example, the antibody from which the CDR residues are derived), for example, to restore or improve antibody specificity or affinity.

[00115] Humanized antibodies and methods of making them are reviewed, for example, in Almagro and Fransson, (2008) *Front. Biosci.* 13: 1619-1633, and are further described, for example, in Riechmann *et al.*, (1988) *Nature* 332:323-329; Queen *et al.*, (1989) *Proc. Natl Acad. Sci. USA* 86: 10029-10033; US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri *et al.*, (2005) *Methods* 36:25-34; Padlan, (1991) *Mol. Immunol.* 28:489-498 (describing “resurfacing”); Dall’Acqua *et al.*, (2005) *Methods* 36:43-60 (describing “FR shuffling”); and Osbourn *et al.*, (2005) *Methods* 36:61-68 and Klimka *et al.*, (2000) *Br. J. Cancer*, 83:252-260 (describing the “guided selection” approach to FR shuffling).

[00116] Human framework regions that can be used for humanization include but are not limited to: framework regions selected using the “best-fit” method (see, for example, Sims *et al.* (1993) *J. Immunol.* 151 :2296); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of heavy chain variable regions (see, for example, Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; and Presta *et al.* (1993) *J. Immunol.* 151:2623); human mature (somatically mutated) framework regions or human germline framework regions (see, for example, Almagro and Fransson, (2008) *Front. Biosci.* 13:1619-1633); and framework regions derived from screening FR libraries (see, for example, Baca *et al.*, (1997) *J. Biol. Chem.* 272: 10678-10684 and Rosok *et al.*, (1996) *J. Biol. Chem.* 271 :22611-22618). Typically, the FR regions of a VHH are replaced with human FR regions to make a humanized VHH. In some embodiments, certain FR residues of the human FR are replaced in order to improve one or more properties of the humanized VHH. VHH domains with such replaced residues are still referred to herein as “humanized.”

[00117] In various embodiments, an Fc region included in a modified IL-2 containing polypeptide is a human Fc region, or is derived from a human Fc region.

[00118] In some embodiments, an Fc region included in a modified IL-2 containing polypeptide is derived from a human Fc region, and comprises a three amino acid deletion in the lower hinge corresponding to IgG1 E233, L234, and L235, herein referred to as “Fc xELL.” Fc xELL polypeptides do not engage FcγRs and thus are referred to as “effector silent” or “effector

null”, however in some embodiments, xELL Fc regions bind FcRn and therefore have extended half-life and transcytosis associated with FcRn mediated recycling.

[00119] In some embodiments, the Fc region included in a modified IL-2 containing polypeptide is derived from a human Fc region and comprises mutations M252Y and M428V, herein referred to as “Fc-YV”. In some embodiments, the Fc region included in a modified IL-2 containing polypeptide is derived from a human Fc region and comprises mutations M252Y and M428L, herein referred to as “Fc-YL”. In some embodiments, such mutations enhance binding to FcRn at the acidic pH of the endosome (near 6.5), while losing detectable binding at neutral pH (about 7.2), allowing for enhanced FcRn mediated recycling and extended half-life.

[00120] In some embodiments, the Fc region included in a modified IL-2 containing polypeptide herein is derived from a human Fc region and comprises mutations designed for heterodimerization, herein referred to as “knob” and “hole”. In some embodiments, the “knob” Fc region comprises the mutation T366W. In some embodiments, the “hole” Fc region comprises mutations T366S, L368A, and Y407V. In some embodiments, Fc regions used for heterodimerization comprise additional mutations, such as the mutation S354C on a first member of a heterodimeric Fc pair that forms an asymmetric disulfide with a corresponding mutation Y349C on the second member of a heterodimeric Fc pair. In some embodiments, one member of a heterodimeric Fc pair comprises the modification H435R or H435K to prevent protein A binding while maintaining FcRn binding. In some embodiments, one member of a heterodimeric Fc pair comprises the modification H435R or H435K, while the second member of the heterodimeric Fc pair is not modified at H435. In various embodiments, the hole Fc region comprises the modification H435R or H435K (referred to as “hole-R” in some instances when the modification is H435R), while the knob Fc region does not. In some instances, the hole-R mutation improves purification of the heterodimer over homodimeric hole Fc regions that may be present.

[00121] Nonlimiting exemplary Fc regions that may be used in a modified IL-2 containing polypeptide include Fc regions comprising the amino acid sequences of SEQ ID NOs: 47-83.

[00122] In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31 and an Fc region fused to the C-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises an amino acid sequence selected from SEQ ID NOs: 3, 5-9, 12-21, and 23-31 and an Fc region fused to the C-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing

polypeptide that comprises at least one antigen binding domain and an Fc region comprises the amino acid sequence of SEQ ID NO: 21 and an Fc region fused to the C-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31 and an Fc region fused to the N-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises an amino acid sequence selected from SEQ ID NOs: 3, 5-9, 12-21, and 23-31 and an Fc region fused to the N-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises the amino acid sequence of SEQ ID NO: 21 and an Fc region fused to the N-terminus of that amino acid sequence. In some embodiments, a modified IL-2 containing polypeptide that comprises at least one antigen binding domain and an Fc region comprises an amino acid sequence selected from SEQ ID NOs: 34-43 and 46. In some embodiments the polypeptide comprises SEQ ID NO: 43 and an antigen binding domain that binds an antigen expressed on a T cell or natural killer cell. In some embodiments, the polypeptide comprises SEQ ID NO: 46 and an antigen binding domain that binds an antigen expressed on a T cell or natural killer cell.

Exemplary activities of modified IL-2 containing polypeptides

[00123] In various embodiments, the modified IL-2 containing polypeptides provided herein are agonists of IL-2R activity. Agonist activity may be determined, in some embodiments, using the methods provided in the Examples herein, such as using 293F cells or similar cells. In some embodiments, the modified IL-2 containing polypeptides provided herein are agonists of IL-2R activity when targeted to T cells, but show little or no agonist activity in the absence of targeting. In some embodiments, the modified IL-2 containing polypeptides provided herein are agonists of IL-2R activity when targeted to NK cells and/or T cells, but show little or no agonist activity in the absence of targeting. In some embodiments, the modified IL-2 containing polypeptides that target T cells or NK cells comprise at least one antigen binding domain that specifically binds to an antigen expressed on T cells or NK cells.

[00124] In some embodiments, the modified IL-2 containing polypeptides provided herein increase proliferation of CD4⁺ and/or CD8⁺ T cells *in vitro* and/ or *in vivo*. In some embodiments, the polypeptide increases CD4⁺ and/or CD8⁺ T cell proliferation in the presence of Treg cells. In some such embodiments, the CD4⁺ and/or CD8⁺ T cells are activated CD4⁺ and/or CD8⁺ T cells. In some embodiments, a modified IL-2 containing polypeptide provided herein increases activated CD4⁺ and/or CD8⁺ T cells proliferation *in vitro*. In some embodiments, the modified IL-2 containing polypeptide increases activated CD4⁺ and/or CD8⁺

T cells proliferation by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold relative to CD4⁺ and/or CD8⁺ T cell proliferation in the absence of the polypeptide. In some embodiments, the polypeptide increases proliferation of activated CD4⁺ and/or CD8⁺ T cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold and does not substantially increase the proliferation of resting CD4⁺ and/or CD8⁺ T cells, relative to the proliferation observed in the absence of the polypeptide.

[00125] In some embodiments, the modified IL-2 containing polypeptides provided herein increase proliferation of NK cells *in vitro* and/ or *in vivo*. In some such embodiments, the NK cells are activated NK cells. In some embodiments, a modified IL-2 containing polypeptide provided herein increases activated NK cells proliferation *in vitro*. In some embodiments, the modified IL-2 containing polypeptide increases activated NK cells proliferation by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold relative to NK cell proliferation in the absence of the polypeptide. In some embodiments, the polypeptide increases proliferation of activated NK cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold and does not substantially increase the proliferation of resting NK cells, relative to the proliferation observed in the absence of the polypeptide.

[00126] The increase in proliferation of activated CD4⁺ and/or CD8⁺ T cells may be determined by any method in the art, such as for example, the methods provided in the Examples herein. A nonlimiting exemplary assay is as follows. CD4⁺ and/or CD8⁺ T cells may be isolated from one or more healthy human donors. The T cells are stained with CellTrace Violet (CTV) and activated with anti-CD3 antibody, contacted with a polypeptide comprising a modified IL-2, and then analyzed by FACS. Loss of CTV staining indicates proliferation. In some embodiments, an increase in CD4⁺ and/or CD8⁺ T cell proliferation is determined as an average from a set of experiments or from pooled T cells, such as by measuring proliferation of CD4⁺ and/or CD8⁺ T cells isolated from different healthy human donors. In some embodiments, an increase in CD4⁺ and/or CD8⁺ T cell proliferation is determined as an average from experiments carried out using T cells from at least five or at least ten different healthy donors, or from a pool of T cells from at least five or at least ten different healthy donors. In some embodiments, the modified IL-2 containing polypeptides provided herein increase proliferation of CD4⁺ and/or CD8⁺ T cells even in the presence of Treg cells.

[00127] In some embodiments, the modified IL-2 containing polypeptides provided herein increase CD71 expression on CD4⁺ and/or CD8⁺ T cells *in vitro* and/ or *in vivo*. CD71 expression indicates T cell activation. In some embodiments, a modified IL-2 containing polypeptide provided herein increases CD71 expression on CD4⁺ and/or CD8⁺ T cells *in vitro*. In some embodiments, the modified IL-2 containing polypeptide increases CD71 expression on

CD4⁺ and/or CD8⁺ T cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold relative to CD71 expression in the absence of the polypeptide. In some embodiments, the polypeptide increases CD71 expression on activated CD4⁺ and/or CD8⁺ T cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold and does not substantially increase CD71 expression on resting CD4⁺ and/or CD8⁺ T cells, relative to the CD71 expression observed in the absence of the polypeptide. In some embodiments, the polypeptide increases CD71 expression on CD4⁺ and/or CD8⁺ T cells in the presence of Treg cells.

[00128] The increase in CD71 expression on CD4⁺ and/or CD8⁺ T cells may be determined by any method in the art, such as for example, the methods provided in the Examples herein. A nonlimiting exemplary assay is as follows. CD4⁺ and/or CD8⁺ T cells may be isolated from one or more healthy human donors and stimulated with an anti-CD3 antibody, contacted with a modified IL-2 containing polypeptide, and then analyzed by FACS for CD71 expression. In some embodiments, an increase in CD71 expression on CD4⁺ and/or CD8⁺ T cells is determined as an average from a set of experiments or from pooled T cells, such as by measuring CD71 expression on CD4⁺ and/or CD8⁺ T cells isolated from different healthy human donors. In some embodiments, an increase in CD71 expression on CD4⁺ and/or CD8⁺ T cells is determined as an average from experiments carried out using T cells from at least five or at least ten different healthy donors, or from a pool of T cells from at least five or at least ten different healthy donors. In some embodiments, the modified IL-2 containing polypeptides provided herein increase CD71 expression on CD4⁺ and/or CD8⁺ T cells even in the presence of Treg cells.

[00129] In some embodiments, the modified IL-2 containing polypeptides provided herein increase pSTAT5 expression in CD4⁺ and/or CD8⁺ T cells *in vitro* and/or *in vivo*. pSTAT5 expression indicates T cell activation. In some embodiments, a modified IL-2 containing polypeptide provided herein increases pSTAT5 expression in CD4⁺ and/or CD8⁺ T cells *in vitro*. In some embodiments, the modified IL-2 containing polypeptide increases pSTAT5 expression on CD4⁺ and/or CD8⁺ T cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold relative to pSTAT5 expression in the absence of the polypeptide. In some embodiments, the polypeptide increases pSTAT5 expression on CD4⁺ and/or CD8⁺ T cells in the presence of Treg cells. The increase in pSTAT5 expression in CD4⁺ and/or CD8⁺ T cells may be determined by any method in the art, such as for example, the methods provided in the Examples herein. In some embodiments, the modified IL-2 containing polypeptides provided herein increase pSTAT5 expression in CD4⁺ and/or CD8⁺ T cells even in the presence of Treg cells.

[00130] In some embodiments, the modified IL-2 containing polypeptides provided herein increase pSTAT5 expression in NK cells *in vitro* and/or *in vivo*. pSTAT5 expression indicates NK cell activation. In some embodiments, a modified IL-2 containing polypeptide provided herein increases pSTAT5 expression in NK cells *in vitro*. In some embodiments, the modified IL-2 containing polypeptide increases pSTAT5 expression on NK cells by at least 1.5-fold, at least 2-fold, at least 3-fold, or by at least 5-fold relative to pSTAT5 expression in the absence of the polypeptide. In some embodiments, the polypeptide increases pSTAT5 expression in NK cells in the presence of Treg cells. The increase in pSTAT5 expression in NK cells may be determined by any method in the art, such as for example, the methods provided in the Examples herein.

[00131] In some embodiments, the modified IL-2 containing polypeptides provided herein reduce or attenuate suppressive activity of regulatory T cells (Tregs). In some embodiments, the modified IL-2 containing polypeptides reduce Treg suppressive activity on CD4⁺ and/or CD8⁺ T cells by at least 10%, at least 20%, at least 30%, or by at least 50%. The decrease in Treg suppressive activity on conventional CD4⁺ and/or CD8⁺ T cells may be determined by any method in the art, such as for example, the methods provided in the Examples herein. A nonlimiting exemplary assay is as follows. Tregs and CD4⁺ T cells are differentially labeled with fluorescent proliferative cellular dyes following isolation from healthy human donor PBMCs. CD4⁺ T cells are stimulated with an anti-CD3 antibody, while Treg cells are incubated in the presence of a modified IL-2 containing polypeptide provided herein. The two T cell populations are co-cultured for 3 days and proliferation and activation of CD4⁺ T cells is monitored by flow cytometry. In some embodiments, the modified IL-2 containing polypeptides provided herein increase CD4⁺ and/or CD8⁺ T cell activation and proliferation in the presence of Treg cells, for example, compared to CD4⁺ and/or CD8⁺ T cell activation and proliferation in the presence of Treg cells but the absence of a modified IL-2 containing polypeptide provided herein.

Polypeptide Expression and Production

[00132] Nucleic acid molecules comprising polynucleotides that encode a modified IL-2 containing binding polypeptide are provided. Thus, in various embodiments, nucleic acid molecules are provided that encode a polypeptide comprising a modified IL-2. In some embodiments, the nucleic acid molecule encodes a modified IL-2 and at least one antigen binding domain. In various embodiments, the nucleic acid molecule encodes a modified IL-2 and an Fc region and, optionally, at least one antigen binding domain. In some embodiments, the Fc region comprises mutations designed for heterodimerization, such as “knob” or “hole” mutations. In some embodiments, a nucleic acid molecule is provided that encodes a modified

IL-2 containing polypeptide that comprises a modified IL-2, at least one antigen binding domain, and an Fc region, wherein the Fc region is fused to the C-terminus of the at least one antigen binding domain, and the modified IL-2 is fused to the C-terminus of the Fc region. In any of the foregoing embodiments, the nucleic acid molecule may also encode a leader sequence that directs secretion of the modified IL-2 containing polypeptide, which leader sequence is typically cleaved such that it is not present in the secreted polypeptide. The leader sequence may be a native heavy chain (or VHH) leader sequence, or may be another heterologous leader sequence.

[00133] Nucleic acid molecules can be constructed using recombinant DNA techniques conventional in the art. In some embodiments, a nucleic acid molecule is an expression vector that is suitable for expression in a selected host cell.

[00134] Vectors comprising nucleic acids that encode the modified IL-2 containing polypeptides described herein are provided. Such vectors include, but are not limited to, DNA vectors, phage vectors, viral vectors, retroviral vectors, *etc.* In some embodiments, a vector is selected that is optimized for expression of polypeptides in a desired cell type, such as 293F, CHO, or CHO-derived cells, or in NSO cells. Exemplary such vectors are described, for example, in Running Deer *et al.*, *Biotechnol. Prog.* 20:880-889 (2004).

[00135] In some embodiments, a modified IL-2 containing polypeptide may be expressed in prokaryotic cells, such as bacterial cells; or in eukaryotic cells, such as fungal cells (such as yeast), plant cells, insect cells, and mammalian cells. Such expression may be carried out, for example, according to procedures known in the art. Exemplary eukaryotic cells that may be used to express polypeptides include, but are not limited to, COS cells, including COS 7 cells; 293 cells, including 293F cells; CHO cells, including CHO-S, DG44, Lec13 CHO cells, and FUT8 CHO cells; PER.C6[®] cells (Crucell); and NSO cells. In some embodiments, the modified IL-2 containing polypeptides may be expressed in yeast. *See, e.g.*, U.S. Publication No. US 2006/0270045 A1. In some embodiments, a particular eukaryotic host cell is selected based on its ability to make desired post-translational modifications to the polypeptide. For example, in some embodiments, CHO cells produce polypeptides that have a higher level of sialylation than the same polypeptide produced in 293F cells.

[00136] Introduction of one or more nucleic acids (such as vectors) into a desired host cell may be accomplished by any method, including but not limited to, calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, *etc.* Nonlimiting exemplary methods are described, for example, in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 3rd ed. Cold Spring

Harbor Laboratory Press (2001). Nucleic acids may be transiently or stably transfected in the desired host cells, according to any suitable method.

[00137] Host cells comprising any of the nucleic acids or vectors described herein are also provided. In some embodiments, a host cell that expresses a modified IL-2 containing polypeptide described herein is provided. The modified IL-2 containing polypeptides expressed in host cells can be purified by any suitable method. Such methods include, but are not limited to, the use of affinity matrices or hydrophobic interaction chromatography. Suitable affinity ligands include the ROR1 ECD and agents that bind Fc regions. For example, a Protein A, Protein G, Protein A/G, or an antibody affinity column may be used to bind the Fc region and to purify a modified IL-2 containing polypeptide that comprises an Fc region. Hydrophobic interactive chromatography, for example, a butyl or phenyl column, may also be suitable for purifying some polypeptides such as antibodies. Ion exchange chromatography (for example anion exchange chromatography and/or cation exchange chromatography) may also be suitable for purifying some polypeptides such as antibodies. Mixed-mode chromatography (for example reversed phase/anion exchange, reversed phase/cation exchange, hydrophilic interaction/anion exchange, hydrophilic interaction/cation exchange, *etc.*) may also be suitable for purifying some polypeptides such as antibodies. Many methods of purifying polypeptides are known in the art.

[00138] In some embodiments, the modified IL-2 containing polypeptide is produced in a cell-free system. Nonlimiting exemplary cell-free systems are described, for example, in Sitaraman *et al.*, *Methods Mol. Biol.* 498: 229-44 (2009); Spirin, *Trends Biotechnol.* 22: 538-45 (2004); Endo *et al.*, *Biotechnol. Adv.* 21: 695-713 (2003).

[00139] In some embodiments, modified IL-2 containing polypeptides prepared by the methods described above are provided. In some embodiments, the modified IL-2 containing polypeptide is prepared in a host cell. In some embodiments, the modified IL-2 containing polypeptide is prepared in a cell-free system. In some embodiments, the modified IL-2 containing polypeptide is purified. In some embodiments, a cell culture media comprising a modified IL-2 containing polypeptide is provided.

[00140] In some embodiments, compositions comprising antibodies prepared by the methods described above are provided. In some embodiments, the composition comprises a modified IL-2 containing polypeptide prepared in a host cell. In some embodiments, the composition comprises a modified IL-2 containing polypeptide prepared in a cell-free system. In some embodiments, the composition comprises a purified modified IL-2 containing polypeptide.

Exemplary methods of treating diseases using modified IL-2 containing polypeptides

[00141] In some embodiments, methods of treating disease in an individual comprising administering a modified IL-2 containing polypeptide are provided. Such diseases include any disease that would benefit from increase proliferation and activation of CD4⁺ and/or CD8⁺ T cells. In some embodiments, methods for treating cancer in an individual are provided. The method comprises administering to the individual an effective amount of a modified IL-2 containing polypeptide provided herein. Such methods of treatment may be in humans or animals. In some embodiments, methods of treating humans are provided. Nonlimiting exemplary cancers that may be treated with modified IL-2 containing polypeptides provided herein include basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; gastrointestinal cancer; glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; liver cancer; lung cancer; small-cell lung cancer; non-small cell lung cancer; adenocarcinoma of the lung; squamous carcinoma of the lung; melanoma; myeloma; neuroblastoma; oral cavity cancer; ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; and vulval cancer; lymphoma; Hodgkin's lymphoma; non-Hodgkin's lymphoma; B-cell lymphoma; low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; Waldenstrom's macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; and chronic myeloblastic leukemia.

[00142] The modified IL-2 containing polypeptides can be administered as needed to subjects. Determination of the frequency of administration can be made by persons skilled in the art, such as an attending physician based on considerations of the condition being treated, age of the subject being treated, severity of the condition being treated, general state of health of the subject being treated and the like. In some embodiments, an effective dose of a modified IL-2 containing polypeptides is administered to a subject one or more times. In some embodiments, an effective dose of a modified IL-2 containing polypeptide is administered to the subject daily, semiweekly, weekly, every two weeks, once a month, etc. An effective dose of a modified IL-2

containing polypeptide is administered to the subject at least once. In some embodiments, the effective dose of a modified IL-2 containing polypeptide may be administered multiple times, including multiple times over the course of at least a month, at least six months, or at least a year.

[00143] In some embodiments, pharmaceutical compositions comprising a modified IL-2 containing polypeptide are administered in an amount effective for treating (including prophylaxis of) cancer and/or increasing T cell proliferation. The therapeutically effective amount is typically dependent on the weight of the subject being treated, his or her physical or health condition, the extensiveness of the condition to be treated, or the age of the subject being treated. In general, polypeptides may be administered in an amount in the range of about 0.05 mg/kg body weight to about 100 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 10 µg/kg body weight to about 100 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 50 µg/kg body weight to about 5 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 100 µg/kg body weight to about 10 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 100 µg/kg body weight to about 20 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 0.5 mg/kg body weight to about 20 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 0.5 mg/kg body weight to about 10 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 0.05 mg/kg body weight to about 20 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 0.05 mg/kg body weight to about 10 mg/kg body weight per dose. In some embodiments, polypeptides may be administered in an amount in the range of about 5 mg/kg body weight or lower, for example less than 4, less than 3, less than 2, or less than 1 mg/kg of the antibody.

[00144] In some embodiments, modified IL-2 containing polypeptides can be administered *in vivo* by various routes, including, but not limited to, intravenous, intra-arterial, parenteral, intraperitoneal or subcutaneous. The appropriate formulation and route of administration may be selected according to the intended application.

[00145] In some embodiments, a therapeutic treatment using a modified IL-2 containing polypeptide is achieved by increasing T cell proliferation and/or activation. In some embodiments, increasing T cell proliferation and/or activation inhibits growth of cancer.

Pharmaceutical compositions

[00146] In some embodiments, compositions comprising modified IL-2 containing polypeptides are provided in formulations with a wide variety of pharmaceutically acceptable carriers (see, for example, Gennaro, Remington: The Science and Practice of Pharmacy with Facts and Comparisons: Drugfacts Plus, 20th ed. (2003); Ansel *et al.*, Pharmaceutical Dosage Forms and Drug Delivery Systems, 7th ed., Lippencott Williams and Wilkins (2004); Kibbe *et al.*, Handbook of Pharmaceutical Excipients, 3rd ed., Pharmaceutical Press (2000)). Various pharmaceutically acceptable carriers, which include vehicles, adjuvants, and diluents, are available. Moreover, various pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are also available. Non-limiting exemplary carriers include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

[00147] In some embodiments, a pharmaceutical composition comprises a modified IL-2 containing polypeptide at a concentration of at least 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, 60 mg/mL, 70 mg/mL, 80 mg/mL, 90 mg/mL, 100 mg/mL, 125 mg/mL, 150 mg/mL, 175 mg/mL, 200 mg/mL, 225 mg/mL, or 250 mg/mL.

Combination Therapy

[00148] Modified IL-2 containing polypeptides can be administered alone or in combination with other modes of treatment, such as other anti-cancer agents. They can be provided before, substantially contemporaneous with, or after other modes of treatment (i.e., concurrently or sequentially). In some embodiments, the method of treatment described herein can further include administering: radiation therapy, chemotherapy, vaccination, targeted tumor therapy, CAR-T therapy, oncolytic virus therapy, cancer immunotherapy, cytokine therapy, surgical resection, chromatin modification, ablation, cryotherapy, an antisense agent against a tumor target, a siRNA agent against a tumor target, a microRNA agent against a tumor target or an anti-cancer/tumor agent, or a biologic, such as an antibody, cytokine, or receptor extracellular domain-Fc fusion.

[00149] In some embodiments, a modified IL-2 containing polypeptide provided herein is given concurrently with a second therapeutic agent, for example, a PD-1 antibody. Examples of PD-1 antibodies include nivolumab (BMS); pembrolizumab (Merck); AMP-514 (Amplimmune); TSR-042 (Tesar/AnaptysBio, ANB-011); STI-A1110 (Sorrento Therapeutics); and other agents that are directed against programmed death-1 (PD-1).

[00150] In some embodiments, a modified IL-2 containing polypeptide provided herein is given concurrently with a second therapeutic agent, for example, a PD-L1 therapy. Examples of PD-L1 therapies include pidilizumab (CureTech, CT-011); durvalumab

(Medimmune/AstraZeneca); atezolizumab (Genentech/Roche); avelumab (Pfizer); AMP-224 (Amplimmune); BMS-936559 (Bristol-Myers Squibb); STI-A1010 (Sorrento Therapeutics); and other agents directed against programmed death-1 ligand (PD-L1).

[00151] In some embodiments, a modified IL-2 containing polypeptide provided herein is given concurrently with CAR-T (chimeric antigen receptor T cell) therapy, oncolytic virus therapy, cytokine therapy, and/or agents that target other checkpoint molecules, such as VISTA, gpNMB, B7H3, B7H4, HHLA2, CD73, CTLA4, TIGIT, etc.

Nonlimiting exemplary methods of diagnosis and treatment

[00152] In some embodiments, the methods described herein are useful for evaluating a subject and/or a specimen from a subject (*e.g.* a cancer patient). In some embodiments, evaluation is one or more of diagnosis, prognosis, and/or response to treatment.

[00153] In some embodiments, the methods described herein comprise evaluating a presence, absence, or level of a protein. In some embodiments, the methods described herein comprise evaluating a presence, absence, or level of expression of a nucleic acid. The compositions described herein may be used for these measurements. For example, in some embodiments, the methods described herein comprise contacting a specimen of the tumor or cells cultured from the tumor with a therapeutic agent as described herein.

[00154] In some embodiments, the evaluation may direct treatment (including treatment with the polypeptides described herein). In some embodiments, the evaluation may direct the use or withholding of adjuvant therapy after resection. Adjuvant therapy, also called adjuvant care, is treatment that is given in addition to the primary, main or initial treatment. By way of non-limiting example, adjuvant therapy may be an additional treatment usually given after surgery where all detectable disease has been removed, but where there remains a statistical risk of relapse due to occult disease. In some embodiments, the polypeptides are used as an adjuvant therapy in the treatment of a cancer. In some embodiments, the antibodies are used as the sole adjuvant therapy in the treatment of a cancer. In some embodiments, the antibodies described herein are withheld as an adjuvant therapy in the treatment of a cancer. For example, if a patient is unlikely to respond to an antibody described herein or will have a minimal response, treatment may not be administered in the interest of quality of life and to avoid unnecessary toxicity from ineffective chemotherapies. In such cases, palliative care may be used.

[00155] In some embodiments the polypeptides are administered as a neoadjuvant therapy prior to resection. In some embodiments, neoadjuvant therapy refers to therapy to shrink and/or downgrade the tumor prior to any surgery. In some embodiments, neoadjuvant therapy means chemotherapy administered to cancer patients prior to surgery. In some embodiments, neoadjuvant therapy means a polypeptide is administered to cancer patients prior to surgery.

Types of cancers for which neoadjuvant chemotherapy is commonly considered include, for example, breast, colorectal, ovarian, cervical, bladder, and lung. In some embodiments, the antibodies are used as a neoadjuvant therapy in the treatment of a cancer. In some embodiments, the use is prior to resection.

[00156] In some embodiments, the tumor microenvironment contemplated in the methods described herein is one or more of: tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor.

Kits

[00157] Also provided are articles of manufacture and kits that include any of the modified IL-2 containing polypeptides as described herein, and suitable packaging. In some embodiments, the invention includes a kit with (i) a modified IL-2 containing polypeptide, and (ii) instructions for using the kit to administer the modified IL-2 containing polypeptide to an individual.

[00158] Suitable packaging for compositions described herein are known in the art, and include, for example, vials (*e.g.*, sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed. Also provided are unit dosage forms comprising the compositions described herein. These unit dosage forms can be stored in a suitable packaging in single or multiple unit dosages and may also be further sterilized and sealed. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine-readable instructions (*e.g.*, instructions carried on a magnetic or optical storage disk) are also acceptable. The instructions relating to the use of the antibodies generally include information as to dosage, dosing schedule, and route of administration for the intended treatment or industrial use. The kit may further comprise a description of selecting an individual suitable or treatment.

[00159] The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. For example, kits may also be provided that contain sufficient dosages of molecules disclosed herein to provide effective treatment for an individual for an extended period, such as about any of a week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, or more. Kits may also include multiple unit doses of molecules and instructions for use and packaged in quantities sufficient for storage and use in pharmacies, for example, hospital pharmacies and compounding

pharmacies. In some embodiments, the kit includes a dry (*e.g.*, lyophilized) composition that can be reconstituted, resuspended, or rehydrated to form generally a stable aqueous suspension of polypeptide.

EXAMPLES

[00160] The examples discussed below are intended to be purely exemplary of the invention and should not be considered to limit the invention in any way. The examples are not intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: P65R mutation of IL-2 essentially eliminates CD25 binding

[00161] IL-2 mutants were designed to disrupt the CD25 interface through steric occlusion (P65R and P65E), and were tested for binding to 293F cells transiently transfected with one or more components of the IL-2 receptor (CD25, CD122, and/or CD132). The mutants were compared to IL-2-F42K, a mutant reported to have reduced affinity to CD25. Increasing concentrations of a fusion protein comprising wild type human IL-2 (SEQ ID NO: 32), IL-2-F42K (SEQ ID NO: 33), IL-2-P65R (SEQ ID NO: 35), or IL-2-P65E (SEQ ID NO: 34) fused to the N-terminus of a “knob” Fc and complexed with a “hole” Fc (SEQ ID NO: 44) were added to the transfected 293F cells and incubated at 4°C for 45 minutes.

[00162] Binding was analyzed by flow cytometry, substantially as follows. Cells were washed once in 200 µL of FACS buffer (PBS, 2% FBS, 0.05% sodium azide) and cell pellets were resuspended in 100 µL of a surface marker staining solution (containing A647-conjugated anti-human Fcγ secondary antibody at 1:300 dilution in FACS buffer). Cells were incubated for 45 minutes at 4°C before the final wash, and analyzed on a flow cytometer. Cellular debris was excluded by FSC/SSC size exclusion, and dead cells were excluded based on their positive propidium iodide signal. Single cells were selected using FSC-A/FSC-H doublet and aggregate exclusion. Transiently transfected cells also expressed cytoplasmic EGFP, and cells that were FL1 positive were analyzed. Increasing MFI levels of anti-human secondary antibody indicated IL-2 binding. FlowJo software was used for analysis of the cell populations. Raw mean fluorescence intensities (“MFI”) for each marker were then exported and analyzed using Excel and GraphPad PRISM. Values were graphed, and titration curves were fitted to assess a dose-response relationship using the non-linear regression One-site -- Total curve fit.

[00163] As shown in FIG. 2A-C, the fusion protein comprising the IL-2-P65E variant exhibited slightly reduced affinity for IL-2R relative to the fusion protein comprising wild type IL-2. The fusion protein comprising IL-2 F42K exhibited lower affinity than the fusion protein comprising IL-2-P65E, while the fusion protein comprising IL-2-P65R exhibited the lowest affinity for heterotrimeric IL-2R (FIG. 2A). Moreover, the fusion protein comprising IL-2-P65R exhibited no detectable binding to CD25/CD132 and only weakly bound CD25/CD122 (FIG. 2C), while the fusion protein comprising IL-2-F42K retained some affinity for CD25/CD132 (FIG. 2B) and bound CD25/CD122 with greater affinity than the fusion protein comprising IL-2-P65R (FIG. 2B and 2C). Thus, IL-2 mutated at P65R significantly reduced binding to CD25 containing IL-2 receptors.

Example 2: IL-2 modifications that reduce affinity for CD122

[00164] As noted in Example 1, the P65R IL-2 mutation was designed to disrupt the CD25 interface through steric occlusion. In addition, IL-2 mutations were designed to reduce affinity for the CD122 interface through elimination of certain contact residue interactions (*e.g.*, D84S, E95Q, M23A, H16A, and E15S). Single or double mutants were fused to the N-terminus of the “knob” half of a heterodimeric Fc (disulfide stabilized knob into hole comprising “hole” Fc SEQ ID NO: 44) for monovalent IL-2 binding to IL-2R. Relative binding affinities were assessed by transiently transfecting 293F cells with CD25 and CD122 (co-transfection with CD132 showed similar results, although the additional binding avidity reduced the differences in affinity observed). Bound IL-2-Fc fusion proteins were detected with fluorescent anti-human secondary antibody and analyzed by flow cytometry, substantially as described in Example 1.

[00165] As shown in FIG. 3A-3B, all of the fusion proteins comprising double IL-2 mutants incorporating F42K with mutations in the CD122 interface (SEQ ID NOs: 36-39 and) showed reduced binding affinity for CD25/CD122 relative to those comprising the single mutant IL-2-F42K (SEQ ID NO: 33), with the exception of IL-2-F42K-E15S (SEQ ID NO: 85).

Example 3: IL-2-RAS (P65R, H16A, and D84S) has reduced affinity for CD122 in the context of trimeric and dimeric forms of IL-2R

[00166] Mutations to reduce CD122 affinity described in Example 2 were combined with the P65R mutation to construct IL-2 double and triple mutants. The IL-2 mutants were fused to the N-terminus of the “knob” half of a heterodimeric Fc and paired with a “hole” Fc comprising SEQ ID NO: 44 for monovalent IL-2 binding to IL-2 receptor (IL-2R). Relative binding affinities of the resulting fusion proteins were assessed on 293F cells transiently transfected with IL-2R subunits, substantially as described in Example 1.

[00167] Relative to the fusion protein comprising wild type IL-2 (SEQ ID NO: 32), the fusion protein comprising IL-2-P65R-H16A (SEQ ID NO: 41) and the fusion protein comprising

IL-2-P65R-D84S (SEQ ID NO: 42) had reduced affinity to both CD122/CD132 (heterodimeric IL-2R) (FIG. 4A) and the heterotrimeric IL-2R (FIG. 4B), while the affinity of the fusion protein comprising triple mutant, IL-2 P65R-H16A-D84S (“IL-2-RAS”, SEQ ID NO: 43), was even more attenuated (FIG. 4A-4B). The shifts in binding observed for these IL-2 mutants in both maximal binding and EC₅₀ suggest that these mutations reduced the on-rate (right shift in EC₅₀) and the off-rate (reduced maximal binding).

Example 4. IL-2-RAS has reduced affinity for resting T cells and pre-activated T cells

[00168] To isolate T cells, non-T cell populations were labeled with biotinylated anti-lineage marker antibodies against CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCR γ/δ (BioLegend) for 20 minutes at room temperature. Non-T cell populations were then depleted by incubating for 20 minutes at room temperature with magnetic streptavidin particles (500 μ l bead slurry plus 500 μ l cell suspension per 100x10⁶, 2x8 minutes incubation on the magnet). The unbound cell supernatant contained isolated T cells.

[00169] Some of the isolated T cells (5.5 x 10⁶ in 3 mL) were activated by incubating in a 6-well plate pre-coated with 1 μ g/ml anti-CD3 OKT3 antibody (BD Biosciences) for 2 days, then washed with PBS/ 2% FBS, and rested at 2x10⁶/mL in RPMI + 10% FBS for 1 day. Resting or pre-activated T cells were used directly in the binding assay. Binding of a non-targeting VHH-Fc isotype control and fusion proteins comprising IL-2-RAS or wild type IL-2 fused to the C-terminus of a non-targeted VHH linked to a heterodimeric Fc to resting or pre-activated T cells was measured by flow cytometry, substantially as described in Example 1 except that the following secondary antibodies were used: AF647 anti-human Fc (1:1000), PI (1:2000), BV785-CD4 (1:300), APC/Fire-CD8 (1:500) and PE/Cy7-CD25 (1:100).

[00170] The non-targeted IL-2-RAS fusion protein (comprising SEQ ID NO: 46) bound with reduced affinity to resting (FIG. 5A) and pre-activated (FIG. 5B) T cells compared to the fusion protein comprising a non-targeting VHH domain and wild-type IL-2 (comprising SEQ ID NO: 45). An isotype control comprising no IL-2 did not bind resting or pre-activated T cells, as shown in FIG. 5A and 5B.

Example 5: IL-2-RAS has reduced affinity for Tregs

[00171] Regulatory T cells (“Tregs”) have high endogenous expression of CD25, as well as of CD122 and CD132, and are highly responsive to wild type IL-2. Binding to Tregs of a fusion protein comprising wild type IL-2 (comprising SEQ ID NO: 45) or the IL-2-RAS triple mutant (comprising SEQ ID NO: 46) fused to the C-terminus of the “knob” half of a heterodimeric Fc (disulfide stabilized knob into hole) of a non-targeted VHH was measured.

[00172] Tregs and CD4⁺ T responder cells (Tresp) were enriched and isolated from fresh, healthy donor PBMCs by using an EasySep Human CD4⁺CD127^{low}CD25⁺ regulatory T cell

isolation kit (Stemcell) following the manufacturer's instructions. Tregs were generated from naive CD4⁺ T cells via 7 day culture in ImmunoCult-XF T Cell Expansion Medium supplemented with rhTGF-B1, all-trans retinoic acid, CD3/CD28 T Cell Activator and IL-2.

[00173] In order to distinguish the two populations of cells, enriched Tregs and CD4⁺ responder T cells were labeled with the proliferative dyes CellTrace Violet (CTV) and CFSE, respectively, for 10 minutes at 37°C. After washing, Tregs and CD4⁺ T cells were resuspended to 1.5x10⁶ cells/ml in RPMI supplemented with 10% FBS and 1X antibiotic/antimycotic. Tregs were seeded in 50 µl volume yielding 75,000 Tregs/well in a 96-well round-bottom plate. Tregs were incubated overnight at 37°C in the presence of 10 nM of IL-2-RAS by flow cytometry as described in Example 1.

[00174] As shown in FIG. 6, in contrast to the fusion protein comprising wild type IL-2, the fusion protein comprising IL-2-RAS showed no observable binding to Tregs enriched from PBMCs (FIG. 6A), induced Tregs (FIG. 6B), or CD4⁺ T responders (FIG. 6C).

Example 6: IL-2-RAS has reduced activity on resting T cells

[00175] T cells were isolated by magnetic bead separation, substantially as described in Example 4, labeled with CellTrace Violet (CTV), and treated with a fusion protein comprising wild type IL-2 (comprising SEQ ID NO: 45) or IL-2-RAS (comprising SEQ ID NO: 46) fused to the C-terminus of a non-targeted VHH linked to a heterodimeric Fc. Levels of CD4, CD8, CD71, and CTV were measured by flow cytometry. Proliferating T cells have reduced CTV levels.

[00176] As shown in FIG. 7A and FIG. 7C, the concentration of the fusion protein comprising IL-2-RAS required to induce resting CD4⁺ and CD8⁺ T cell proliferation was over 100 times greater than the concentration of a fusion protein comprising wild type IL-2 or the concentration of a fusion protein comprising IL-2v-analog required to achieve the same induction of proliferation.

[00177] As shown in FIG. 7B and FIG. 7D, the concentration of the fusion protein comprising IL-2-RAS required to induce CD71 expression, a marker of T cell activation, on CD8⁺ and CD4⁺ T cells, was at least 100 times greater than the concentration of the fusion protein comprising wild type IL-2 or IL-2v-analog required to achieve the same induction of activation.

[00178] T cell activation can also be measured by phosphorylated STAT5 levels, which are increased in activated T cells. T cells were isolated by magnetic bead separation and treated with the fusion protein comprising wild-type IL-2 (comprising SEQ ID NO: 45) fused to the C-terminus of a non-targeted VHH comprising a heterodimeric Fc or the fusion protein comprising IL-2-RAS (comprising SEQ ID NO: 46) fused to the C-terminus of a non-targeted VHH comprising a heterodimeric Fc for 15 minutes. Cells were fixed with BD Cytofix/Cytoperm™

(BD Biosciences), permeabilized in 90% ice-cold methanol, and levels of phosphorylated STAT5 ("pSTAT5") on CD4⁺ and CD8⁺ T cells were measured using flow cytometry using an anti-pSTAT5-PE antibody (1:70). Cells were co-stained with the following antibodies: anti-CD3-FITC (1:200), CD56-BV421 (1:100), CD4-BV785 (1:200), CD8-APC-Fire (1:300).

[00179] As shown in FIG. 7E and FIG. 7F, the non-targeted IL-2-RAS fusion protein achieved minimal phosphorylation of STAT5 in resting CD4⁺ and CD8⁺ T cells even at the highest concentration tested, while the non-targeted IL-2-wild type fusion protein induced STAT5 phosphorylation at a concentration more than 1000 times less than the highest concentration tested.

Example 7: IL-2 mutants have reduced activity on Tregs

[00180] Tregs were isolated from PBMCs using the EasySep™ Human CD4⁺CD127^{low}CD25⁺ Regulatory T cell Isolation Kit (Stemcell). Tregs were labeled with CellTrace Violet and plated at 0.15x10⁶ cells per well (96-well, U-bottom) in 100 µl of RPMI/10% FBS. Cells were combined with 100 µl of a fusion protein titration starting at 100nM, titrated 1:4. Cells were incubated for 7 days. On day 7, proliferation and activation marker CD25 were measured by flow cytometry (Novocyte) substantially as described in Example 1, except that the following antibodies were used: BV785-CD4 (1:300), APC/Fire-CD8 (1:500) PE/Cy7-CD25 (1:100), PI (1:2000).

[00181] As shown in FIG. 8A and 8B, fusion protein comprising wild type IL-2 (comprising SEQ ID NO: 45) fused to the C-terminus of a non-targeted VHH linked to heterodimeric Fc, but not fusion protein comprising IL-2-RAS in place of wild type IL-2 (comprising SEQ ID NO: 46), induced Treg proliferation and expression of the activation marker CD25.

Example 8: Activated T cells expressing PD-1 are stimulated by PD-1-targeted IL-2-RAS

[00182] The ability to bind to and stimulate PD-1 expressing T cells was tested using pembrolizumab (an anti-PD-1 conventional antibody) and a fusion protein comprising a pembrolizumab analog and IL-2-RAS linked to the C-terminus of the heavy chain (see FIG. 1F).

[00183] Enriched T cells from a healthy donor were activated, substantially as described in Example 4. 6-well plates were coated overnight with 1 µg/ml OKT3 antibody at 4°C. The next day, plates were washed two times to remove unbound OKT3 antibody. Enriched T cells were thawed using CTL media and resuspended to 5.5 x 10⁶ cells/mL in complete RPMI and seeded in 3 mL per well in the coated plates. Two days later, the activated T cells were collected and washed once before plating in media without OKT3 antibody for 24 hours to rest. Cells were labeled with the proliferative dye CellTrace™ Violet (CTV). The T cells were counted,

then resuspended to 2×10^6 cells/mL. 100 μ L of resuspended cells were seeded per well in a 96-well round-bottom plate. Pembrolizumab or a pembrolizumab analog-IL-2-RAS fusion was added starting at a final concentration of 100 nM and titrated 1:5. On day three, T cells were stained for 20 min at room temperature with the viability marker PI and the following fluorescently labeled antibodies: CD4-BV785, CD8-APC/Fire, CD25-PE/Cy7, CD71-FITC, and CD69-APC. The plate was read on the Novocyte flow cytometer substantially as described in Example 7 for measurement of proliferation and as in Example 1 for binding and data was exported into Excel for further analysis.

[00184] As shown in FIG. 9, the pembrolizumab analog-IL-2-RAS fusion protein stimulated CD8⁺ T cell proliferation (FIG. 9A) and CD4⁺ T cell proliferation (FIG. 9B), while pembrolizumab alone did not. Without intending to be bound by any particular theory, the biphasic nature of the observed proliferation may suggest that the activity at low concentration is due to PD-1-targeted activity and the increased activity at higher concentration is due to non-targeted activity. As shown in FIG. 9C and 9D, both pembrolizumab and pembrolizumab analog-IL-2-RAS bound activated CD8⁺ and CD4⁺ T cells with similar affinities, except that additional binding was observed for the fusion protein comprising IL-2-RAS at the upper end of the dilution range above 10nM, which may have been mediated by IL-2-RAS binding to IL-2R.

Example 9: Pre-blocking PD-1 on activated T cells prevents signaling by PD-1 targeted IL-2-RAS

[00185] T cells were isolated and enriched from a healthy donor by magnetic bead separation, and incubated on plates coated with OKT3 antibody to activate them, substantially as described in Example 4. The cells were labeled with CTV. The pre-activated T cells were incubated with pembrolizumab, an anti-PD-1 antibody, to block PD-1 binding sites, or a non-targeted antibody as a control. The cells were then incubated with a fusion protein comprising IL-2-RAS fused to a pembrolizumab analog, or a fusion protein comprising IL-2-RAS fused to a non-targeting antibody as a control, for 3 days. The extent of IL-2 signaling was evaluated by measuring CD4⁺ and CD8⁺ T cell proliferation by flow cytometry, substantially as described in Example 7.

[00186] As shown in FIG. 10A-10D, wild type IL-2 induced robust proliferation of both CD8⁺ and CD4⁺ T cells, while CD4⁺ T cells and CD8⁺ T cells treated with pembrolizumab or the fusion protein comprising IL-2-RAS and the non-targeting antibody exhibited low levels of proliferation that was not affected by pre-blocking of PD-1. In contrast, both CD4⁺ T cells (FIG. 10B and 10D) and CD8⁺ T cells (FIG. 10A and 10C) treated with the fusion protein comprising IL-2-RAS and a pembrolizumab analog exhibited significant PD-1 dependent proliferation (FIG. 10A and 10B), which was blocked by pre-incubation with an anti-PD-1 antibody (FIG. 10C and

10D). Thus, a fusion protein comprising IL-2-RAS and an anti-PD-1 antibody activated T cells only when PD-1 was both expressed and accessible on the T cells.

Example 10: PD-1-targeted IL-2-RAS overcomes Treg suppression

[00187] CD4⁺ T responder cells and Tregs were isolated as described in Example 5. The CD4⁺ responder cells were labeled with CTV, mixed with isolated Tregs at a ratio of 2:1 and activated with anti-CD3 beads (1 bead per 2 T cells). The resulting mixture was treated with a dilution series of a wild type IL-2 fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B, a fusion protein comprising IL-2-RAS fused to the C-terminus of a non-targeted VHH, as shown in FIG. 1B, or with a fusion protein comprising IL-2-RAS fused to an anti-PD-1 antibody (pembrolizumab analog-IL-2-RAS) for 7 days. Proliferation was measured by flow cytometry, substantially as described in Example 7.

[00188] As shown in FIG. 11, Trespander cells were suppressed by Tregs, but non-targeted wild type IL-2 and the fusion protein comprising IL-2-RAS and an anti-PD-1 antibody (pembrolizumab analog-IL-2-RAS) induced CD4⁺ T responder cell proliferation despite the presence of Tregs. Treating cells with a fusion protein comprising IL-2-RAS and a non-targeted antibody did not rescue proliferation to a similar extent. The non-targeted IL-2-RAS was only able to counter the suppressive effects of Tregs on Tresponders at much higher concentrations than the PD-1 targeted IL-2-RAS fusion protein.. Thus, PD-1-targeted IL-2-RAS overcame the suppressive effects of Tregs, and this activity was dependent on binding PD-1 expressed on the T cells.

Example 11: PD-1 targeted IL-2-RAS does not signal in trans

[00189] Beads are coated with 200 µg PD-1 antigen per 4 x 10⁸ beads according to the manufacturer's recommended coating procedure. In brief, beads are washed once in buffer 1 (0.1 M sodium phosphate buffer, pH 7.4–8.0) and then incubated in a tube rotator for 18 hours at room temperature in buffer 1 containing PD-1 antigen. Beads are then washed 4 times with buffer 2 (PBS, 0.1% BSA, 2 mM EDTA pH 7.4). Free tosyl groups are deactivated by incubation of beads for 4 hours at 37°C in buffer 3 (0.2 M Tris, 0.1% BSA, pH 8.5). Beads are then washed once in buffer 2 and resuspended to a concentration of 400x10⁶ beads/mL.

[00190] Coated beads are incubated with a fusion protein comprising wild typeIL-2 or IL-2-RAS fused to an anti-PD-1 antibody and washed. The beads are then incubated with isolated resting T cells. IL-2 signaling is evaluated by measuring pSTAT5 levels via flow cytometry.

[00191] The fusion protein comprising wild typeIL-2 bound to the beads robustly activates CD8⁺ T cells and CD4⁺ T cells, while the fusion protein comprisingIL-2-RAS bound to the beads has no activity up to the highest concentration tested on either CD4⁺ or CD8⁺ T

cells. Thus, T cell targeting of IL-2-RAS is required for IL-2 signaling, and signaling of targeted IL-2-RAS does not occur in trans.

Example 12: IL-2-RAS does not signal in trans

[00192] Dilution series of non-targeted wild type IL-2 and of non-targeted IL-2-RAS, starting at 1000 nM and diluted 1:4, were coated on assay plates, incubated overnight, and washed. T cells were added and incubated at 37 °C for 30 minutes. Activation of CD8⁺ and CD4⁺ T cells was measured by detecting phosphorylated STAT5 levels, substantially as described in Example 6.

[00193] As shown in FIG. 12A and 12B, CD8⁺ and CD4⁺ T cells were activated by wild type IL-2 in trans, as measured by pSTAT5 induction; however, non-targeted IL-2-RAS was unable to activate in trans. Without intending to be bound by any particular theory, the reduced affinities of IL-2-RAS for CD25 and CD122 may have prevented efficient binding and clustering of the IL-2R to induce downstream signaling. Thus, only targeted IL-2-RAS fusion proteins drive pSTAT5 signaling.

Example 13: NKp46 targeted IL-2-RAS specifically drives NK cell proliferation

[00194] The effects of a fusion protein comprising IL-2-RAS fused to the C-terminus of a heterodimeric scFv antibody targeting NKp46, as shown in FIG. 1H, fusion proteins comprising wild type IL-2 or IL-2-RAS fused to the C-terminus of a non-targeted VHH linked to a heterodimeric Fc, as shown in FIG. 1B, and the heterodimeric scFv antibody targeting NKp46 alone on NK cells, CD4⁺ T cells, and CD8⁺ T cells were determined.

[00195] Fresh PBMCs from a healthy donor were labeled with CellTrace™ Violet and plated in a 96-well round bottom plate at 200,000 cells/well. Dilutions of the fusion proteins and NKp46 scFv-Fc control were added to the plated cells and incubated at 37 °C for 7 days. On day 7, cell proliferation was measured, substantially as described in Example 7, except that the following antibodies were used: anti-CD3-BV785 (1:200), anti-CD56-APC (1:100), anti-CD4-PE (1:200), anti-CD8-APC-Fire (1:300) and PI (1:2000).

[00196] In addition, fresh PBMCs from a healthy donor were treated with the same fusions proteins or NKp46 scFv-Fc control, and incubated at 37°C for 15 minutes. pSTAT5 levels in CD8⁺ T cells, CD4⁺ T cells, and NK cells (CD3⁻, CD56⁺) were measured by detecting phosphorylated STAT5 levels, substantially as described in Example 6.

[00197] Binding of the fusion proteins and the NKp46 scFV-Fc control to fresh PBMCs from a healthy donor was measured, substantially as described in Example 1, except that the following antibodies were used: anti-CD3-FITC (1:100), anti-CD56-BV421 (1:100), anti-CD4-BV785 (1:200), anti-CD8-APC-Fire (1:300), anti-human IgG-Alexa Fluor 647 (1:500), and PI (1:2000).

[00198] As shown in FIG. 13A-13I, NKp46-targeted IL-2-RAS potently activated NK cell proliferation and activation, while not affecting CD4⁺ or CD8⁺ T cells. In contrast, non-targeted wild type IL-2 drove proliferation and activation of all lymphocytes tested (NK, CD4⁺, and CD8⁺ T cells). Binding of the NKp46 scFV-Fc (without IL-2-RAS) did not drive NK proliferation or pSTAT5 induction. Thus, NKp46-targeted IL-2-RAS drove cis signaling of IL-2 on NK cells, but did not activate CD4⁺ or CD8⁺ T cells in trans.

Example 14: LAG3 targeted IL-2-RAS stimulates pre-activated LAG3⁺ T-cells

[00199] The effects on CD4⁺ T cells and CD8⁺ T cells of fusion proteins comprising IL-2-RAS fused to the C-terminus of an anti-LAG3 heterodimeric conventional antibody (MAb), as shown in FIG. 1G, fused to an anti-LAG3 VHH with an heterodimeric Fc as shown in FIG. 1B, fused to a non-targeted VHH, as shown in FIG. 1B, or a fusion protein comprising wild type IL-2 fused to the C-terminus of a non-targeted heterodimeric Fc, as shown in FIG. 1B, or a LAG3-targeted Mab (control), or a LAG3-targeted VHH-Fc (control) were assayed.

[00200] Enriched T cells from a healthy donor were stimulated for 48 hours with 1 µg/mL coated anti-CD3 (OKT3) and 10 µg/mL soluble anti-CD28, then allowed to rest for 24 hours. The pre-activated cells were labeled with CellTrace™ Violet and seeded at 200,000 cells/well. Dilutions of the fusion proteins and control proteins were added and incubated for 3 days. Proliferation and expression of activation markers CD25 and CD71 were measured, substantially as in Example 7, but with these additional antibodies: anti-CD25-FITC (1:100) and anti-CD71-PE/Cy7.

[00201] Stimulated CD8⁺ T cells upregulated LAG3 to 45% of CD8⁺ T cells, while CD4⁺ T cells upregulated LAG3 to 22% of CD4⁺ T cells. In contrast, non-stimulated T cells are close to 0% positive for LAG3 expression on either CD8⁺ or CD4⁺ T cells.

[00202] As shown in FIG. 14A-14D, both anti-LAG3 Mab-IL-2-RAS and anti-LAG3 VHH-IL-2-RAS increased CD8⁺ and CD4⁺ proliferation (FIG. 14A and 14B) and activation as indicated by CD25 (FIG. 14C and 14D) and CD71 (FIG. 14E and 14F) expression levels. Non-targeted wild type IL-2 was a strong inducer of CD8⁺ and CD4⁺ T cell proliferation and activation, and bound stimulated T cells with higher affinity and saturation.

Example 15: Combination mutants of IL-2 further reduce non-targeted activity

[00203] HEK-Blue IL-2 reporter cells (InvivoGen) were used to measure the relative activities of non-targeted IL-2 mutants. Reporter cells were treated with dilutions of IL-2-mutants fused to the C-terminus of a non-targeted VHH and incubated for 20 hours before Quanti-Blue analysis.

[00204] As shown in FIG. 15, the IL-2 mutants showed a range of activities. Experiments described above showed that IL-2-RAS (P65R, H16A, and D84S) had dramatically reduced binding to IL-2Rs compared to wild type IL-2 (*see* FIG. 4-6), and reduced activity compared to

wild type IL-2 (*see* FIG. 7A-7E). IL-2-RAS with an additional M23A mutation and IL-2-RAS with an additional E95Q mutation both showed reduced activity compared to IL-2-RAS, and the combination of IL-2-RAS with both M23A and E95Q had even further attenuated activity. In experiments with PD-1-expressing reporter cells, these reduced affinity IL-2 mutants all showed comparable PD-1-targeted activity (data not shown), suggesting that high affinity binding to PD-1 in cis can compensate for reduced affinity of IL-2 mutants to IL-2R. While the HEK-Blue IL-2 reporter system was useful for relative activity measurement, the observed EC₅₀ for IL-2 mutants in the reporter system was shifted significantly to the left compared to primary lymphocytes, likely due to the overexpression of IL-2R components in the reporter cell compared to lower IL-2R levels on primary cells.

[00205] The disclosure may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting of the disclosure. Scope of the disclosure is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced herein.

Table of Certain Sequences

SEQ ID NO	Description	Sequence
1	Wild type Human IL-2	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
2	IL-2v	AP[A]SSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T[A][K]FAMPKKATELKHLQC L E E E L K P L E E V L N[G]A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F[A]Q S I I S T L T
3	IL-2-P65R	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K[R]L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
4	IL-2-H16A	APTSSSTKKTQLQLE[A]ALLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
5	IL-2-D84S	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R[S]L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
6	IL-2-E15S	APTSSSTKKTQLQ[L]SHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
7	IL-2-M23A	APTSSSTKKTQLQLEHLLLDLQ[A]I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
8	IL-2-E95Q	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L[Q]L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
9	IL-2-P65E	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K[E]L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
10	IL-2-F42K	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
11	IL-2-H16A-F42K	APTSSSTKKTQLQLE[A]ALLLDLQMI L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
12	IL-2-D84S-F42K	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R[S]L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
13	IL-2-E15S-F42K	APTSSSTKKTQLQ[L]SHLLLDLQMI L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
14	IL-2-M23A-F42K	APTSSSTKKTQLQLEHLLLDLQ[A]I L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
15	IL-2-E95Q-F42K	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T[K]K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L[Q]L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
16	IL-2-P65R-H16A	APTSSSTKKTQLQLE[A]ALLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K[R]L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
17	IL-2-P65R-D84S	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K[R]L E E V L N L A Q S K N F H L R P R[S]L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T

18	IL-2-P65R-E15S	APTSSSTKKTQLQLS ¹ HLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
19	IL-2-P65R-M23A	APTSSSTKKTQLQLEHLLLDLQ ¹ A I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
20	IL-2-P65R-E95Q	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L ³ Q L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
21	IL-2-P65R-H16A-D84S (IL-2-RAS)	APTSSSTKKTQLQLE ¹ A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R ³ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
22	IL-2-T3A-C125S	AP ¹ A S S S T K K T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ² S Q S I I S T L T
23	IL-2-T3A-P65R-C125S	AP ¹ A S S S T K K T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ³ S Q S I I S T L T
24	IL-2-T3A-H16A-C125S	AP ¹ A S S S T K K T Q L Q L E ² A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ³ S Q S I I S T L T
25	IL-2-T3A-D84S-C125S	AP ¹ A S S S T K K T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R ³ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ² S Q S I I S T L T
26	IL-2-T3A-H16A-P65R-C125S	AP ¹ A S S S T K K T Q L Q L E ² A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ³ R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ⁴ S Q S I I S T L T
27	IL-2-T3A-P65R-D84S-C125S	AP ¹ A S S S T K K T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R ³ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ⁴ S Q S I I S T L T
28	IL-2-T3A-H16A-P65R-D84S-C125S	AP ¹ A S S S T K K T Q L Q L E ² A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ³ R L E E V L N L A Q S K N F H L R P R ⁴ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ⁵ S Q S I I S T L T
29	IL-2-no9-H16A-P65R-C125S	T Q L Q L E ¹ A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ³ S Q S I I S T L T
30	IL-2-no9-P65R-D84S-C125S	T Q L Q L E H L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R ³ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ⁴ S Q S I I S T L T
31	IL-2-no9-H16A-P65R-D84S-C125S	T Q L Q L E ¹ A L L L D L Q M I L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K ² R L E E V L N L A Q S K N F H L R P R ³ S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F ⁴ S Q S I I S T L T
32	Wild type IL-2-xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V

		TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALH NHYTQKSLSLSPGK
33	IL-2-F42K- xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
34	IL-2-P65E- xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K E L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
35	IL-2-P65R- xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K R L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
36	IL-2-F42K- D84S-xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R S L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
37	IL-2-F42K- E95Q-xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L Q L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
38	IL-2-F42K- M23A- xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQAI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
85	IL-2-F42K- E15S-xELL “knob” Fc	APTSSSTKKTQLQLSHLLLDLQMI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T P G G G G D K T H T C P P C P A P G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P C R D E L T K N Q V S L W C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
39	IL-2-H16A- F42K-	APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T K K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E

	xELL “knob” Fc	FLNRWITFCQSIISTLTTPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
40	IL-2-H16A- xELL “knob” Fc	APTSSSTKKTQLQLEALLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLC LEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVE FLNRWITFCQSIISTLTTPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
41	IL-2-P65R- H16A-xELL “knob” Fc	APTSSSTKKTQLQLEALLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLC LEELKRLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVE FLNRWITFCQSIISTLTTPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
42	IL-2-P65R- D84S-xELL “knob” Fc	APTSSSTKKTQLQLEHLLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLC LEELKRLEEVLNLAQSKNFHLRPRSLISNINVIVLELKGSETTFMCEYADETATIVE FLNRWITFCQSIISTLTTPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
43	IL-2-RAS- xELL “knob” Fc	APTSSSTKKTQLQLEALLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLC LEELKRLEEVLNLAQSKNFHLRPRSLISNINVIVLELKGSETTFMCEYADETATIVE FLNRWITFCQSIISTLTTPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
44	Linker-EVN xELL “hole” Fc	KPGGGGDKTHTCPPCPAPGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPEVKF NRYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
45	xELL “knob” Fc- IL-2-T3G- C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGSGGSAPGS SSTKKTQLQLEHLLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEEE LKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR WITFSQSIISTLT
46	xELL “knob” Fc- IL-2-RAS- T3G-C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGSGGSAPGS SSTKKTQLQLEALLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEEE LKRLEEVLNLAQSKNFHLRPRSLISNINVIVLELKGSETTFMCEYADETATIVEFLNR WITFSQSIISTLT
47	Fc region 1 (human wild type IgG1)	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
48	Fc region 2 (human IgG1 xELL	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

	“knob”)	
49	Fc region 3 (human IgG1 EVN xELL “hole” I253R)	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQG NVFSCSVMH EALHNHYTQK SLSLSPGK
50	Fc region	DKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK
51	Fc region xELL	DKTHTC PPCPAPGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK
52	Fc region xELL H435R	DKTHTC PPCPAPGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNRYTQK SLSLSPGK
53	Fc region xELL M252Y and M428V (YV)	DKTHTC PPCPAPGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVH EALHNHYTQK SLSLSPGK
54	Fc region xELL M252Y and M428L (YL)	DKTHTC PPCPAPGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVLH EALHNHYTQK SLSLSPGK
55	Fc region xELL M252Y, M428L, H435R (YLR)	DKTHTC PPCPAPGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVLH EALHNRYTQK SLSLSPGK
56	Fc region xELL M252Y, M428V, H435R (YVR)	DKTHTC PPCPAPGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVH EALHNRYTQK SLSLSPGK
57	Fc region xELL S354C T366W knob	DKTHTC PPCPAPGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPCRDELTKNQVSLWCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK
58	Fc region xELL	DKTHTC PPCPAPGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY

	H435R S354C T366W knob	KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPCRDELT KNQVSLWCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNRYTQK SLSLSPGK
59	Fc region xELL M252Y and M428V (YV) S354C T366W knob	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLYSKLTVDKSRWQQGNVFSCSVHEALHNHYTQKSLSLSPGK
60	Fc region xELL M252Y and M428L (YL) S354C T366W knob	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHNHYTQKSLSLSPGK
61	Fc region xELL M252Y, M428L, H435R (YLR) S354C T366W knob	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHNRYTQKSLSLSPGK
62	Fc region xELL M252Y, M428V, H435R (YVR) S354C T366W knob	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLYSKLTVDKSRWQQGNVFSCSVHEALHNRYTQKSLSLSPGK
63	Fc region xELL T366S, L368A, Y407V hole	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVCTLPSSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
64	Fc region xELL H435R, T366S, L368A, Y407V hole	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVCTLPSSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPV LDS DGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRYTQKSLSLSPGK

65	Fc region xELL M252Y and M428V (YV) T366S, L368A, Y407V hole	DKHTCPCPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLTCLVKGFSYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLVSKLTVDKSRWQQG NVFSCSVVHEALHNHYTQKSLSLSPGK
66	Fc region xELL M252Y and M428L (YL) T366S, L368A, Y407V hole	DKHTCPCPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLTCLVKGFSYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLVSKLTVDKSRWQQG NVFSCSVLHEALHNHYTQKSLSLSPGK
67	Fc region xELL M252Y, M428L, H435R (YLR) T366S, L368A, Y407V hole	DKHTCPCPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLTCLVKGFSYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLVSKLTVDKSRWQQG NVFSCSVLHEALHNRYTQKSLSLSPGK
68	Fc region xELL M252Y, M428V, H435R (YVR) T366S, L368A, Y407V hole	DKHTCPCPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLTCLVKGFSYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLVSKLTVDKSRWQQG NVFSCSVVHEALHNRYTQKSLSLSPGK
69	Fc region H435R	DKHTCPCPCP APELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNRYTQK SLSLSPGK
70	Fc region M252Y and M428V (YV)	DKHTCPCPCP APELLGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVVH EALHNHYTQK SLSLSPGK
71	Fc region M252Y	DKHTCPCPCP APELLGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELTKNQVSLTCLV

	and M428L (YL)	KGFYPSDIAV EWESNGQPEN NYKTTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVLH EALHNHYTQK SLSLSPGK
72	Fc region M252Y, M428L, H435R (YLR)	DKTHTCPPCP APELLGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVLH EALHNRYTQK SLSLSPGK
73	Fc region M252Y, M428V, H435R (YVR)	DKTHTCPPCP APELLGGPS VFLFPPKPKD TLYISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVVH EALHNRYTQK SLSLSPGK
74	Fc region S354C T366W knob	DKTHTCPPCP APELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPCRDELT KNQVSLWCLV KGFYPSDIAV EWESNGQPEN NYKTTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK
75	Fc region H435R S354C T366W knob	DKTHTCPPCP APELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPCRDELT KNQVSLWCLV KGFYPSDIAV EWESNGQPEN NYKTTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNRYTQK SLSLSPGK
76	Fc region M252Y and M428L (YL) S354C T366W knob	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKT TPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHNHYTQKSLSLSPGK
77	Fc region M252Y, M428L, H435R (YLR) S354C T366W knob	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKT TPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHNRYTQKSLSLSPGK
78	Fc region M252Y, M428V, H435R (YVR) S354C T366W knob	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKT TPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVVHEALHNRYTQKSLSLSPGK

79	Fc region T366S, L368A, Y407V hole	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT SKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
80	Fc region H435R, T366S, L368A, Y407V hole	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT SKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRYTQKSLSLSPGK
81	Fc region M252Y and M428V (YV) T366S, L368A, Y407V hole	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT SKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVVHEALHNHYTQKSLSLSPGK
82	Fc region M252Y and M428L (YL) T366S, L368A, Y407V hole	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT SKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVLHEALHNHYTQKSLSLSPGK
83	Fc region M252Y, M428L, H435R (YLR) T366S, L368A, Y407V hole	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT SKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVLHEALHNRYTQKSLSLSPGK
84	Truncated wild-type human IL-2	QLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F C Q S I I
86	xELL-Knob Fc-IL2- T3A, C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK KAKGQPREPQVYTLPPQ RDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGSGGSAPAS SSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L N R W I T F S Q S I I S T L T

87	xELL-Knob Fc-IL2- RAS-T3A, C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPP[C]RDELTKNQVSL[W]CLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDSGSGFLYSLKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGGSGGSAP[A]S SSTKKTQLQLEA[ALLLDLQMI]LNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEE LK[R]LEEVLNLAQSKNFHLRPR[S]LISNINVIVLELKGSETTFMCEYADETATIVEFLNR WITF[S]QSIISTLT
88	Pembrolizu mab analog Knob Fc- IL2-RAS- T3A, C125S	QVQLVQSGVEVKKPGASVKVSCASGYTFTNYYMYWVRQAPGQGLEWMGGINPSNGGT NFNEKFKNRVTLTDSSTTTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGQGTTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV YTLPP[C]RDELTKNQVSL[W]CLVKGFYPSDIAVEWESNGQPENNY[D]TTPVLDSDGSGFL YS[D]LTVDKSRWQQGNVFSCSVMHEALHN[R]YTQKSLSLSP[GGSGGS]AP[A]SSSTKKTQL QLEA[ALLLDLQMI]LNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEEELK[R]LEEVL NLAQSKNFHLRPR[S]LISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF[S]QSI ISTLT
89	Pembrolizu mab analog Hole Fc	QVQLVQSGVEVKKPGASVKVSCASGYTFTNYYMYWVRQAPGQGLEWMGGINPSNGGT NFNEKFKNRVTLTDSSTTTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGQGTTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV [C]TLPPSRD[K]LTKNQVSL[S]CA[V]KGFYPSDIAVEW[K]SNGQPENNYKTTTPVLDSDGSGF L[V]SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
90	Pembrolizu mab Light Chain analog	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWYQQKPGQAPRLLIYLYSL ESGVPARFSGSGSDTFTLTISSELEPEDFAVYYCQHSRDPLTFGGGTKEIKRTVAA PSVFI FPPSDEQLKSGTASVVCLLNMFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
91	Pembrolizu mab analog IL2-RAS- T3G, C125S	QVQLVQSGVEVKKPGASVKVSCASGYTFTNYYMYWVRQAPGQGLEWMGGINPSNGGT NFNEKFKNRVTLTDSSTTTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGQGTTV TVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVTVPSSSLGTQTYTCNVDHKPSNTKVDKRVESKYGPCCPPCAP EFLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSDQEDPEVQFNWYVDGVEVHNAKTK PREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSGFFL YSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSP[GGSGGS]AP[G]SSSTKKTQLQ LEA[ALLLDLQMI]LNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEEELK[R]LEEVLN LAQSKNFHLRPR[S]LISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF[S]QSI ISTLT
92	NKp46- scFv xELL- Knob Fc- IL2-RAS- T3A, C125S	QVQLQQSGPELVKPGASVKMSCKASGYTFTDYVINWGKQSRGQGLEWIGEIPGSGTN YYNEKFKAATLTADKSSNIAYMQLSSLTSEDSAVYFCARRGRYGLYAMDYWGQTSV TVSS[VEGGSGSGSGSGSGGVD]DIQMTQTSSLSASLGDRVTISCRASQDISNYLNWY QQKPDGTVKLLIYYTSRLHSGVPSRFSGSGSGTDYSLTINNLEQEDIATYFCQQGNTR PWTFGGGTKLEIKP[GGG]DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAPIEKTISKAKGQPREPQVYTLPP[C]RDELTKNQVSL[W]CLVKGFYPSDIA VEWESNGQPENNY[D]TTPVLDSDGSGFFLYS[D]LTVDKSRWQQGNVFSCSVMHEALHN[R]Y TQKSLSLSP[GGSGGS]AP[A]SSSTKKTQLQLEA[ALLLDLQMI]LNGINNYKNPKLTRMLTFKF YMPKKATELKHLQCLEEEELK[R]LEEVLNLAQSKNFHLRPR[S]LISNINVIVLELKGSETT FMCEYADETATIVEFLNRWITF[S]QSIISTLT
93	NKp46- scFv xELL- Hole Fc	QVQLQQSGPELVKPGASVKMSCKASGYTFTDYVINWGKQSRGQGLEWIGEIPGSGTN YYNEKFKAATLTADKSSNIAYMQLSSLTSEDSAVYFCARRGRYGLYAMDYWGQTSV TVSS[VEGGSGSGSGSGSGGVD]DIQMTQTSSLSASLGDRVTISCRASQDISNYLNWY QQKPDGTVKLLIYYTSRLHSGVPSRFSGSGSGTDYSLTINNLEQEDIATYFCQQGNTR PWTFGGGTKLEIKP[GGG]DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAPIEKTISKAKGQPREPQV[C]TLPPSRD[K]LTKNQVSL[S]CA[V]KGFYPSDIA

		VEW[K]SNGQPENNYKTTTPVLDS[K]GSFFL[V]SKLTVDKSRWQQGNVFSCSVMEALHNHY TQKSLSLSPGK
94	NKp46- scFv xELL- Fc	QVQLQQSGPELVKPGASVKMSCKASGYTFTDYVINWGKQRSGQGLEWIGEIYPGSGTN YYNEKFKAKATLTADKSSNIAYMQLSSLTSEDSAVYFCARRGRYGLYAMDYWGQGTSTV TVSSVEGGSGGSGGSGGSGGVDDI[Q]MTQTTSLSASLGDRVTISCRASQDISNYLNWY QQKPDGTVKLLIYYTSRLHSGVPSRFSGSGSGTDYSLTINNLEQEDIATYFCQQGNTR PWTFGGGTKLEIKPGGGGDKHTCPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTTPVLDSGSGFFLYSKLTVDKSRWQQGNVFSCSVMEALHN HYTQKSLSLSPGK
95	LAG3-MAb xELL-Knob Fc-IL2- RAS-TGCS	QVQLVQSGAEVKKPGASVKVSCCKASGYTFTGYMHVWRQAPGQGLEWMGWINANSNGGT NYAQKFQGRVTMTDRDTSISTAYMELSRRLSDDTAVYYCARDIYDSSDQLNVWGQGTMTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPP[C]RDELTKNQVSL[W]CLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSG FFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP[GGSGGS]AP[GS]SSTKKT QLQLE[A]LLLDLQMI LINGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEELK[R]LEE VLNLAQSKNFHLRPR[S]LISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF[S]Q SIISTLT
96	LAG3-MAb xELL-Hole Fc	QVQLVQSGAEVKKPGASVKVSCCKASGYTFTGYMHVWRQAPGQGLEWMGWINANSNGGT NYAQKFQGRVTMTDRDTSISTAYMELSRRLSDDTAVYYCARDIYDSSDQLNVWGQGTMTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPGGPSVFLFPPKPKDTLM[R]SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV [C]TLPPSRDELTKNQVSL[S]CA[V]KGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSGFFL [V]SKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGK
97	LAG3-MAb Light Chain	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRAITGI PARFSGSGSGTDFTLTITSSLEPEDFAVYYCQQASIWPLTFGGGTKEIKRTVAAPSVF IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYS LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
98	LAG3-MAb IgG1	QVQLVQSGAEVKKPGASVKVSCCKASGYTFTGYMHVWRQAPGQGLEWMGWINANSNGGT NYAQKFQGRVTMTDRDTSISTAYMELSRRLSDDTAVYYCARDIYDSSDQLNVWGQGTMTV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSG FFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGK
99	LAG3-VHH xELL-Knob Fc-IL2- RAS-TGCS	EVQLVESGGGWQPGGSLRLSCAASGRFTSDYVMGWFRQAPGKEREFAVAISESGGRTH YADSVKGRFTISRDN SKNTLYLQMN SLRPEDTALYYCATLLWWTSEYAPIKANDYDY WGQGTLLVTVKP[GGGG]DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPP[C]RDELTKNQVSL[W]CLVKGFYPSDIAVEWE SNGQPENNYKTTTPVLDSGSGFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKS LSLSP[GGSGGS]AP[GS]SSTKKTQLQLE[A]LLLDLQMI LINGINNYKNPKLTRMLTFKFYMP KKATELKHLCLEELK[R]LEEVNLAQSKNFHLRPR[S]LISNINVIVLELKGSETTFMC EYADETATIVEFLNRWITF[S]QSIISTLT
100	LAG3-VHH xELL- Hole_H435 R Fc	EVQLVESGGGWQPGGSLRLSCAASGRFTSDYVMGWFRQAPGKEREFAVAISESGGRTH YADSVKGRFTISRDN SKNTLYLQMN SLRPEDTALYYCATLLWWTSEYAPIKANDYDY WGQGTLLVTVKP[GGGG]DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQV[C]TLPPSRDELTKNQVSL[S]CA[V]KGFYPSDIAVEWE SNGQPENNYKTTTPVLDSGSGFFL[V]SKLTVDKSRWQQGNVFSCSVMEALHN[R]YTQKS LSLSPGK
101	LAG3-VHH xELL-Knob	EVQLVESGGGWQPGGSLRLSCAASGRFTSDYVMGWFRQAPGKEREFAVAISESGGRTH YADSVKGRFTISRDN SKNTLYLQMN SLRPEDTALYYCATLLWWTSEYAPIKANDYDY WGQGTLLVTVKP[GGGG]DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS

	Fc	NKALPAPIEKTISKAKGQPREPQVYTLPPC[CRDELTKNQVSLW]CLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKS LSLSPGK
102	xELL-Knob Fc-IL2- RAS- M23A-T3A, C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP[GGSGGS]AP[AS SSTKKTQLQLE[ALLLDLQ[AILNGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEEE LK[RL]EEVLNLAQSKNFHLRPR[SLISNINVIVLQ]LKGETTFMCEYADETATIVEFLNR WITF[SQSIISTLT
103	xELL-Knob Fc-IL2- RAS-E95Q- T3A, C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP[GGSGGS]AP[AS SSTKKTQLQLE[ALLLDLQ[AILNGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEEE LK[RL]EEVLNLAQSKNFHLRPR[SLISNINVIVLQ]LKGETTFMCEYADETATIVEFLNR WITF[SQSIISTLT
104	xELL-Knob Fc-IL2- RAS- M23A- E95Q-T3A, C125S	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP[GGSGGS]AP[AS SSTKKTQLQLE[ALLLDLQ[AILNGINNYKNPKLTRMLTFKFYMPKKATELKHLCLEEE LK[RL]EEVLNLAQSKNFHLRPR[SLISNINVIVLQ]LKGETTFMCEYADETATIVEFLNR WITF[SQSIISTLT

[00206] In sequences that contain boxes or underlining, the boxes around individual letters indicate amino acid substitutions relative to a corresponding wild type or parental sequence; boxes around groups of letters indicate linker sequences. Underlined letters are linker sequences.

What is claimed is:

1. A polypeptide comprising a modified IL-2, wherein the modified IL-2 comprises a P65R substitution, a H16A substitution, and a substitution at amino acid position D84, wherein the amino acid positions correspond to the amino acid positions in SEQ ID NO: 1.
2. The polypeptide of claim 1, wherein the modified IL-2 is a modified human IL-2.
3. The polypeptide of claim 1, wherein the substitution at amino acid position D84 is selected from D84S, D84G, D84A, D84T, D84V, and D84P.
4. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises a substitution at amino acid position M23, optionally wherein the substitution is selected from M23A, M23G, M23S, M23T, M23V, and M23P.
5. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises a substitution at amino acid position E95, optionally wherein the substitution is selected from E95Q, E95G, E95S, E95T, E95V, E95P, E95H, and E95N.
6. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises a substitution at amino acid position F42, optionally wherein the substitution is selected from F42K, F42A, F42R, F42A, F42G, F42S, and F42T.
7. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises at least one substitution at at least one amino acid position selected from Y45 and L72, optionally wherein the modified IL-2 comprises at least one substitution selected from Y45A and L72G.
8. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises substitutions P65R, H16A, and D84S; P65R, H16A, D84S, and M23A; P65R, H16A, D84S, and E95Q; or P65R, H16A, D84S, M23A, and E95Q.
9. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises at least one substitution at at least one amino acid position selected from T3 and C125, optionally wherein the modified IL-2 comprises at least one substitution selected from T3A and C125A.
10. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises a set of substitutions selected from T3A-H16A-P65R-D84S-C125S; T3A-H16A-M23A-P65R-D84S-C125S; T3A-H16A-P65R-D84S-E95Q-C125S, and T3A-H16A-M23A-P65R-D84S-E95Q-C125S.

11. The polypeptide of any one of claims 8-10, wherein the modified IL-2 comprises the set of substitutions, and does not comprise any additional substitutions.

12. The polypeptide of any one of the preceding claims, wherein the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 84; or wherein the modified IL-2 comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31; or wherein the modified IL-2 comprises an amino acid sequence selected from SEQ ID NOs: 3-9, 11-21, and 23-31.

13. The polypeptide of any one of the preceding claims, wherein the polypeptide comprises an Fc region, optionally wherein the modified IL-2 is fused to the N-terminus or the C-terminus of the Fc region.

14. The polypeptide of claim 13, wherein the Fc region comprises a substitution at Kabat amino acid position T366 or wherein the Fc region comprises at least one substitution at at least one Kabat amino acid position selected from T366, L368, and Y407; optionally wherein the Fc region comprises a T366W substitution or wherein the Fc region comprises T366S, L368A, and Y407V mutations.

15. The polypeptide of claim 13 or claim 14, wherein the Fc region comprises a substitution at a Kabat position selected from S354 and Y349, optionally wherein the Fc region comprises a S354C or a Y349C substitution.

16. The polypeptide of any one of claims 13-15, wherein the Fc region comprises a substitution at Kabat amino acid position H435, optionally wherein the Fc region comprises a substitution selected from H435R and H435K.

17. The polypeptide of any one of claims 13-16, wherein the Fc region comprises at least one substitution at one Kabat amino acid position selected from M252 and M428, optionally wherein the Fc region comprises M252Y and M428V substitutions.

18. The polypeptide of any one of claims 13-17, wherein the Fc region comprises a deletion of Kabat amino acids E233, L234, and L235; or wherein the Fc region comprises at least one substitution at at least one amino acid position selected from L234, L235, and P329, optionally wherein the Fc region comprises L234A, L235A, and P329G substitutions.

19. The polypeptide of any one of claims 13-18, wherein the Fc region comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 47-83.

20. The polypeptide of any one of claims 13-18, wherein the Fc region is part of a heavy chain constant region, optionally wherein the heavy chain constant region is an IgG1, IgG2, IgG3, or IgG4 constant region.

21. The polypeptide of any one of claims 13-20, wherein the modified IL-2 is fused to the C-terminus of the Fc region or heavy chain constant region via a linker comprising 1-20 amino acids, optionally wherein the linker comprises glycine amino acids or glycine and serine amino acids.

22. The polypeptide of any one of claims 13, 14, 18, and 21, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 87, 102, 103, or 104.

23. The polypeptide of any one of the preceding claims, wherein the polypeptide comprises at least one antigen binding domain, or wherein the polypeptide comprises two, three, or four antigen binding domains.

24. The polypeptide of claim 23, wherein at least one antigen binding domain specifically binds to a T-cell antigen or a natural killer cell antigen.

25. The polypeptide of claim 23 or claim 24, wherein at least one antigen binding domain specifically binds to a CD4⁺ T-cell antigen or a CD8⁺ T-cell antigen, optionally wherein the at least one antigen binding domain specifically binds to an antigen on an activated CD4⁺ T-cell or an activated CD8⁺ T-cell.

26. The polypeptide of any one of claims 23-25, wherein at least one antigen binding domain specifically binds to PD-1, CTLA-4, LAG3, TIM3, 4-1BB, OX40, GITR, CD8a, CD8b, CD4, NKp30, NKG2A, TIGIT, TGFβR1, TGFβR2, Fas, NKG2D, NKp46, PD-L1, CD107a, ICOS, TNFR2, or CD16a.

27. The polypeptide of any one of claims 23-26, wherein at least one or each antigen binding domain is a human or humanized antigen binding domain.

28. The polypeptide of any one of claims 23-27, wherein at least one or each antigen binding domain comprises a VHH domain.

29. The polypeptide of any one of claims 23-27, wherein at least one antigen binding domain comprises a VH domain and a VL domain, optionally wherein at least one antigen

binding domain comprises the VH domain and the VL domain of an antibody selected from pembrolizumab, nivolumab, AMP-514, TSR-042, STI-A1110, ipilimumab, tremelimumab, urelumab, utomilumab, atezolizumab, and durvalumab.

30. The polypeptide of claim 29, wherein the at least one antigen binding domain comprises a single chain Fv (scFv).

31. The polypeptide of claim 29, wherein the polypeptide comprises a heavy chain constant region, wherein the VH domain is fused to the heavy chain constant region, and wherein the VL domain is associated with the VH domain, optionally wherein the VL domain is fused to a light chain constant region, optionally wherein the light chain constant region is selected from kappa and lambda.

32. The polypeptide of any one of claims 23-31, wherein each of the antigen binding domains are the same, and/or wherein each of the antigen binding domains specifically binds to the same antigen.

33. The polypeptide of any one of claims 23-32, wherein at least one antigen binding domain specifically binds to PD-1 and at least one other antigen binding domain specifically binds to a T-cell antigen or natural killer cell antigen other than PD-1.

34. The polypeptide of any one of claims 13-33, wherein the polypeptide forms a homodimer under physiological conditions.

35. The polypeptide of any one of claims 1-34, wherein the modified IL-2 binds a human IL-2R with an affinity at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than the affinity of human wild type IL-2 for the IL-2R.

36. A complex comprising a first polypeptide and a second polypeptide, wherein the first polypeptide is the polypeptide of any one of claims 1-35.

37. The complex of claim 36, wherein the first polypeptide comprises a first Fc region and the second polypeptide comprises a second Fc region, optionally wherein each Fc region is an isotype selected from human IgG1, IgG2, IgG3, and IgG4.

38. The complex of claim 36 or claim 37, wherein each Fc region comprises a deletion of amino acids E233, L234, and L235; and/or wherein each Fc region comprises a H435R or H435K mutation; and/or wherein each Fc region comprises a mutations M252Y and M428L or mutations M252Y and M428V; and/or wherein the first Fc region or the second Fc

region comprises a T366W mutation, and the other Fc region comprises mutations T366S, L368A, and Y407V; and/or wherein the first Fc region or the second Fc region comprises a S354C mutation.

39. The complex of any one of claims 36-38, wherein each Fc region independently comprises an amino acid sequence at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to an amino acid sequence selected from SEQ ID NOs: 47-83.

40. The complex of any one of claims 36-39, wherein the second polypeptide does not comprise a modified IL2.

41. The complex of any one of claims 36-40, wherein the first polypeptide comprises a first antigen binding domain, an Fc region, and a modified IL-2 and the first antigen binding domain is fused to the N-terminus of the Fc region and the modified IL-2 is fused to the C-terminus of the Fc region and the second polypeptide comprises a second antigen binding domain and an Fc region.

42. The complex of claim 41, wherein:

- a) the first antigen binding domain and the second antigen binding domain both bind PD-1;
- b) the first antigen binding domain binds PD-1, and the second antigen binding domain binds LAG3;
- c) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CTLA-4;
- d) the first antigen binding domain binds PD-1, and the second antigen binding domain binds 4-1BB;
- e) the first antigen binding domain binds PD-1, and the second antigen binding domain binds OX40;
- f) the first antigen binding domain binds PD-1, and the second antigen binding domain binds GITR;
- g) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD8a;
- h) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD8b;

- i) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD4;
- j) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKp30;
- k) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKG2A;
- l) the first antigen binding domain binds PD-1, and the second antigen binding domain binds TIGIT;
- m) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKG2D;
- n) the first antigen binding domain binds PD-1, and the second antigen binding domain binds TGFBR2;
- o) the first antigen binding domain binds PD-1, and the second antigen binding domain binds Fas;
- p) the first antigen binding domain binds PD-1, and the second antigen binding domain binds CD107a;
- q) the first antigen binding domain binds PD-1, and the second antigen binding domain binds NKp46;
- r) the first antigen binding domain binds CD8a, and the second antigen binding domain binds TGFR β R2;
- s) the first antigen binding domain binds CD8a, and the second antigen binding domain binds Fas;
- t) the first antigen binding domain binds NKG2D, and the second antigen binding domain binds TGFR β R2;
- u) the first antigen binding domain binds NKG2D, and the second antigen binding domain binds Fas;
- v) the first antigen binding domain binds NKG2A, and the second antigen binding domain binds TGFR β R2;
- w) the first antigen binding domain binds NKG2A, and the second antigen binding domain binds Fas;

- x) the first antigen binding domain binds NKp46, and the second antigen binding domain binds TGFR β R2;
- y) the first antigen binding domain binds NKp46, and the second antigen binding domain binds Fas;
- z) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds LAG3;
- aa) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds Tim3;
- bb) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds OX40;
- cc) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds GITR;
- dd) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds CD107a;
- ee) the first antigen binding domain binds CTLA-4, and the second antigen binding domain binds NKp46; or
- ff) the first antigen binding domain binds ICOS, and the second antigen binding domain binds TNFR2.

43. A pharmaceutical composition comprising a polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42 and a pharmaceutically acceptable carrier, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

44. An isolated nucleic acid that encodes a polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42.

45. An expression vector comprising the nucleic acid of claim 44.

46. An isolated host cell comprising the nucleic acid of claim 44 or the expression vector of claim 45.

47. An isolated host cell that expresses the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42.

48. A method of producing the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42 comprising incubating the host cell of claim 46 or claim 47 under conditions suitable to express the polypeptide or complex, and optionally isolating the polypeptide or complex.

49. A method of increasing CD4+ and/or CD8+ T cell proliferation comprising contacting T cells with the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42, wherein the CD4+ and/or CD8+ T cells are *in vitro*, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

50. Use of the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42 in the manufacture of a medicament for increasing CD4+ and/or CD8+ T cell proliferation *in vivo*, wherein the T cells are to be contacted with the medicament, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

51. An *in vitro* method of increasing NK cell proliferation comprising contacting NK cells with the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

52. Use of the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42 in the manufacture of a medicament for increasing NK cell proliferation *in vivo*, wherein the NK cells are to be contacted with the medicament, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

53. Use of the polypeptide of any one of claims 1-35 or the complex of any one of claims 36-42, or the pharmaceutical composition of claim 43 in the manufacture of a medicament for treating a subject with cancer, wherein the substitution at amino acid position D84 is D84S, and wherein the polypeptide or complex comprises at least one antigen binding domain that specifically binds to a T-cell antigen or a natural killer cell antigen.

54. The use of claim 53, wherein the cancer is selected from basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; gastrointestinal cancer; glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; liver cancer; lung cancer; small-cell lung cancer; non-small cell lung cancer; adenocarcinoma of the lung; squamous carcinoma of the lung; melanoma; myeloma; neuroblastoma; oral cavity cancer; ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma; Hodgkin's lymphoma; non-Hodgkin's lymphoma; B-cell lymphoma; low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; Waldenstrom's macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; and chronic myeloblastic leukemia.

55. The use of claim 53 or 54, wherein an additional therapeutic agent is to be administered, optionally wherein the additional therapeutic agent is an anti-cancer agent.

56. The use of claim 55, wherein the anti-cancer agent is selected from a chemotherapeutic agent, an anti-cancer biologic, radiation therapy, CAR-T therapy, and an oncolytic virus, optionally wherein the anti-cancer biologic is an antibody or a cytokine.

57. The use of claim 55 or claim 56, wherein the additional therapeutic agent is an anti-cancer biologic that inhibits PD-1 and/or PD-L1, or inhibits VISTA, gpNMB, B7H3, B7H4, HHLA2, CTLA4, or TIGIT.

58. The use of any one of claims 53-57, wherein the subject is to be further treated with tumor resection and/or radiation therapy.